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MERCURY COMPOUNDS IN THE CHEMOTHERAPY OF EXPERIMENTAL TUBERCULOSIS IN GUINEA-PIGS. I

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XXI

LYDIA M. DEWITT

From the Otho S. A. Sprague Memorial Institute and the Pathological Laboratory of the University of Chicago

Perhaps no drug has a longer history of use in therapy than has mercury. Much of this history, and especially that of the newer organic mercury compounds, has been reviewed by Schamberg, Kolmer and Raiziss [1] and by others, and need not receive further attention here. In tuberculosis, mercury in various forms and by various methods has been used to a greater or less extent clinically since the time of Paracelsus early in the sixteenth century. There have been certain periods when it was in especial favor and others when it was in disrepute. Hall,[2] while not regarding it as a specific, thinks that small doses of mercurial preparations are "our most potent chemical weapons against this disease." Many others at about the same period, published favorable reports on mercury therapy of tuberculosis. In 1908 and 1909 Barton L. Wright [3] reported a considerable number of cases treated with mercuric succinimide and potassium iodide alternately, and his favorable reports were followed by many others, who used the same or similar treatment. The general consensus of opinion seems to be that mercurials have a good effect on the condition of the patient, but that mercury is in no sense a specific in tuberculosis.

Only one report in the literature has been found by me on the use of mercury in experimental tuberculosis in animals. George Cornet [4] tested mercuric chloride among a series of disinfectants, in 8 guinea-pigs. He injected mercuric chloride for 10 days until the animals began to show toxic effects; he then inoculated them with tubercle bacilli. He was unable to note any difference in the extent

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[1] Am. J. Syphilis, 1917, 1, p. 1.
[2] Am. J. Med. Sc., 1889, 98, p. 45.
[3] New York Med. Jour., 1909, 89, p. 1180.
[4] Ztschr. f. Hyg. u. Infektionskr., 1889, 98, p. 58.

of the disease between the mercurialized animals and the controls. Some died of mercurial poisoning early, and all the others showed generalized tuberculosis.

Robert Koch [5] stated that mercury in vapor form inhibited the growth of the tubercle bacillus in the test tube but was entirely without influence on the progress of the disease in animals.

In view of the long continued and repeatedly favorable use of mercurials in the clinical treatment of human tuberculosis and the paucity of experimental evidence as to their value, it seemed advisable, in connection with the systematic investigations on the chemotherapy of tuberculosis carried on in this laboratory, that the value of mercury and its compounds should be investigated in considerable detail.

In the internal use of compounds of mercury, it is necessary to consider (1) the stability of the preparation in the animal tissues, (2) its method and form of absorption and excretion, (3) the place of deposition of the part of the compounds which is not excreted, and (4) the toxicity and pathogenicity of the preparations.

Morley [6] states (1) that French workers claim that mercurials are converted in the stomach and intestines into mercuric chloride and circulate as the double salt of mercury and sodium; (2) that it is proved that mercury is absorbed and escapes as an albuminate in every excretion of the body, especially in the urine; (3) that a single dose is excreted within 24 hours; (4) that it accumulates, if given in small doses, and is deposited in all the organs. Blumenthal and Oppenheim [7] state that after the organic compounds of mercury used by them, a deposit of mercury was found constantly in the liver; sometimes in the intestines and occasionally in the lungs and in the blood. Schamberg, Kolmer and Raiziss [8] state (1) that the toxicity of mercurial salts is directly proportionate to the amount of mercury contained, the differences of molecular structure being of relatively little importance as affecting their toxicity; (2) that insoluble preparations injected intramuscularly are absorbed at the rate of a little over 1% per day; (3) that after 6 or 7 weeks, almost 50% of the mercury of insoluble preparations may be unabsorbed at the site of injection; and (4) that mercury has a great affinity for the cells of the kidney, and that this organ is one of the earliest involved in mercurial intoxication. All other workers do not agree with Schamberg, Kolmer and Raiziss on the relation of molecular structure to toxicity.

The bactericidal action of mercury has long been recognized, but its use as an internal antiseptic has been limited by its toxicity. Hence the efforts of chemists have for years been turned largely to the construction of mercuric compounds which should be nontoxic or less toxic than mercuric chloride, which kills, on the average, in intravenous doses above 4 mg. per kilogram of body weight, according to Sansum.[9] Abelin [10] states that the toxic influ-

[5] Vehr. d. X Internat. Med. Kong., 1890-91, I, Berlin. klin. Wchnschr., 1890, 27, p. 736.
[6] Calif. State Med. Jour., 1909, 7, p. 338.
[7] Biochem. Ztschr., 1914, 65, p. 460.
[8] Boston Med. & Surg. Jour., 1915, 162, p. 826.
[9] Jour. Am. Med. Assn., 1918, 70, p. 824.
[10] Deutsch. med. Wcnschr., 1912, 38, p. 1822.

ence of mercury compounds is in certain relation with their chemical structure, that the toxicity can be diminished by introduction of sulpho or sulph-amino groups or through double carbon connection of mercury, and that easily ionizable mercury compounds are more toxic than the less easily ionizable. Schrauth and Schoeller[11] showed that the disinfectant powers of organic mercury compounds were increased by substitution of the less acid phenolic hydroxyl for the carboxyl. They also showed that of the three isomeric mercuriated cresols, the meta derivative is the most potent disinfectant, while the ortho-hydroxymercuriphenoxide is more active than the para compound. Also, the entrance of a second hydroxymercuri group increases disinfectant power.

In the work, then, with new mercury compounds it has been necessary to keep the following factors in mind:

1. Minimal toxicity for animal tissues, i. e., organotropism.
2. Maximal disinfectant power
3. Stability or minimal ionization
4. Solubility in nontoxic and nonirritant solvents

This report, which will be followed by others on the same subject, concerns the bacteriostatic and therapeutic effect of some twenty-four inorganic and organic compounds of mercury on the human tubercle bacillus and on experimental tuberculosis in guinea-pigs.

Table 1 gives a list of the compounds reported in this paper, with their chemical formulae, when this was available, and their computed percentage content of mercury.

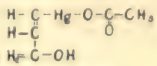
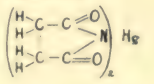
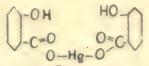
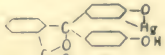
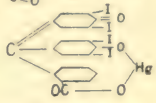
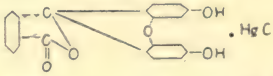
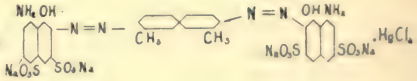
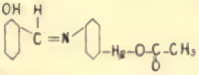
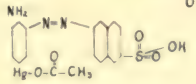
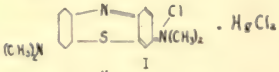
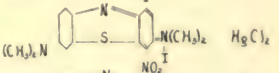
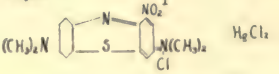
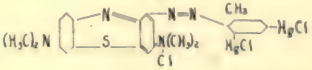
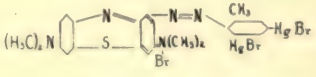
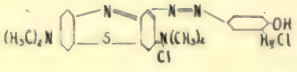
Part of these compounds have been purchased from Merck and Company, while many have been made for me by the following assistants working in the chemical laboratory of the University of Chicago: Walter Frankel, C. E. Cutler, S. M. Cadwell, Gladys Leavell, L. M. Larsen, Morris Kharasch, Friedrich Lommen, I. M. Jacobsohn.

The inhibitory or bacteriostatic power of most of these mercurials was tested in the usual way by adding the required amount of stock solution of the compound to tubes of melted agar and shaking until well mixed; the tubes were then slanted and cooled and inoculated with human tubercle bacilli, control tubes being made from the same culture. Certain of the compounds could not be dissolved in hot or cold water or in hot agar. Some of these could be dissolved in dilute alkali, in which case the stock solutions were made in either normal sodium hydroxide or 5% sodium carbonate (anhydrous) and diluted with water twenty times. The same amount of the alkali was also added to the control tubes to determine whether the alkalinity used had any inhibitory effect on the growth of the organisms. Some of the compounds, as shown in column 2 of table 2, were insoluble in any known solvent that could be used in this experiment. The required

[11] Ztschr. f. Hyg. u. Infektionskr., 1916, 82, p. 279.

TABLE 1

List of Compounds

Name of Compound	Percentage of Mercury	Chemical Formula
Mercurous Chloride	84.92	$(HgCl)_2$
Mercuric Chloride	93.78	$HgCl_2$
Mercuric Sulpho-Cyanide	63.29	$Hg(S-C\equiv N)_2$
Mercury Potassium Cyanide	68.72	$Hg(CN)_2 . 2\,KCN$
Allyl Alcohol Mercuric Acetate	63.3	
Mercuric Succinimide	50.5	
Mercuric Salicylate	42.2	
Mercuro-Iodo Hemol (Merck)	12.0	Patented Preparation of Hemoglobin, Mercury and Iodin
Mercurol (Merck)	10.0	Patented Preparation of Mercury and Nucleinic Acid
Mercury Phenolphthalein	39.0	
Mercury Tetraiodphenolphthalein	19.6	
Fluorescein Mercuric Chloride	33.17	
Trypan Blue Mercuric Chloride	15.65	
Ortho-Oxy-Benzylidene Amino Phenyl-para Mercuric Acetate	44.0	
1-Amino-2 [para-Naphthalin-Azophenyl Mercuric Acetate] 5 Sulphonic Acid	34.2	
Methylene Blue-Mercuric Chloride	28.94	
Iod-Methylene Blue Mercuric Chloride	24.75	
Methylene Green Mercuric Chloride	31.6	
Diazo-Amino Methylene Blue ortho-Toluidin di Mercuric Chloride	29.7	
Diazo-Amino Methylene Blue Brom Hydrate ortho-Toluidin Mercuric Bromide	39.5	
Diazo-Amino Methylene Blue ortho Phenol Mercuric Chloride	29.7	

amount of such insoluble compounds was weighed into each tube of melted agar. The tubes were well shaken and cooled quickly in ice water so that time was not given for the drug to settle out. In this way a fine suspension of the drug in agar was used, and, as can be seen from table 2, such fine suspensions showed quite as great bacteriostatic power as did the true solutions. It is probable that this is due to a slight unrecognizable solubility, so that in a dilution of 1:20,000 the compounds were really in solution. Only in the case of iodo-mercuro-hemol did even this method fail to give good results. When the hot melted agar was added to the powdered mercuro-iodo-hemol, it formed large, coarse, flocculent masses, which would not break up again. This probably explains the fact that only a dilution of 1:1,000 completely inhibited while a dilution of 1:5,000 partially inhibited growth and that there was good growth on all the higher dilutions. Hence, it required 0.12 mg. of mercury in the form of mercuro-iodo-hemol per c c of agar for complete inhibition of growth, far the largest amount required in any of the tests. Mercuric chloride stood next in the amount of mercury per c c needed for inhibition. This seemed surprising, as mercuric chloride is usually considered high in bacteriostatic power. However, I found it impossible to prepare my agar tubes of mercuric chloride without a certain amount of precipitate in the bottom of the tubes. This consisted of metallic mercury, and, of course, considerably reduced the percentage of mercuric chloride in the agar. However, Schamberg and his co-workers [1] also found that many of their organic mercury compounds had a much higher germicidal power than did mercuric chloride, and in their tests the dilutions were made in distilled water. The calculations of the amount of mercury per c c were made in order to determine whether the inhibitory action on the growth of the tubercle bacillus depends solely on the proportionate amount of pure mercury in the molecule, as Schamberg [8] found was true of the toxicity, or whether, as Schamberg [1] found in his bactericidal studies with B. typhosus and Staph. aureus, "the antiseptic value is not related to the amount of pure mercury in the substance, but rather to the chemical constitution of the molecule." By examining the last column of table 2, it may be seen that 4 compounds are, in proportion to the mercury content, considerably higher than the others in bacteriostatic power. These are in order: (1) allyl alcohol mercuric acetate, (2) double salt of methylene blue and mercuric chloride, (3) fluorescein and mercuric chloride and (4) 1- amino, 2 (paranaphthalin azophenyl mercuric acetate) 5 sulphonic acid. The second and

third are double salts of mercuric chloride and a dye which alone has considerable inhibitory power. This is especially true of methylene blue. In the first, mercury is bound to a carbon of an alcohol ring, while in number 4, mercury is bound by one bond to a carbon of a phenyl ring. The double salt of the dye trypan blue and mercuric chloride requires over twice as much mercury per c c to inhibit as the double salt of methylene blue and mercuric chloride, but the dye trypan blue has itself practically no inhibitory power. It seems necessary to conclude from these findings that the different constituents of the molecule play their own rôle in the bacteriostatic action of the mercury compounds. The question of the relation of the position of the mercury in the molecule to bacteriostatic and bactericidal action will be discussed in another paper.

TABLE 2

Bacteriostatic Power

Mercurial	Soluble in	Growth in Tubes								Mg. Hg per C c Needed to Inhibit
		1: 1000	1: 5000	1: 10,000	1: 20,000	1: 50,000	1: 100,000	1: 500,000	Control	
Mercuric chloride	Water	None	None	None	None	Good	Good	Good	Good	0.093
Mercury sulphocyanide	Insoluble, good suspension	None	None	None	None	Good	Good	Good	Good	0.031
Mercury potassium cyanide	Water	None	None	None	None	Good	Good	Good	Good	0.034
Allyl alcohol mercuric acetate	Dilute alkali	None	None	None	None	None	Very slight	Slight	Good	0.012
Mercuric succinimide	Water	None	None	None	None	Good	Good	Good	Good	0.025
Mercuric salicylate	Insoluble, good suspension	None	None	None	None	Good	Good	Good	Good	0.021
Mercuro-iodo-hemol	Insoluble, poor suspension	None	Slight	Good	Good	Good	Good	Good	Good	0.12
Mercurol (mercuric nucleinate)	Water	None	None	Slight	Good	Good	Good	Good	Good	0.02
Mercury phenolphthalein	Dilute alkali	None	None	None	Good	Good	Good	Good	Good	0.039
Fluorescein mercuric chloride	Dilute alkali	None	None	None	None	Good	Good	Good	Good	0.016
Ortho-oxybenzylidene amino phenyl para mercuric acetate	Dilute alkali	None	None	None	None	Good	Good	Good	Good	0.022
1-amino-2(p-naphthalin azophenyl mercuric acetate) - 5 sulphonic acid	Dilute alkali	None	None	None	None	Slight	Slight	Very slight	Good	0.017
Trypan blue mercuric chloride	Slightly in hot water	None	None	Good	Good	Good	Good	Good	Good	0.03
Methylene blue mercuric chloride	Insoluble, good suspension	None	None	None	None	Slight	Good	Good	Good	0.014
Iod methylene blue mercuric chloride	Insoluble. good suspension	None	None	None	Very slight	Good	Good	Good	Good	0.024

It will be seen that all the compounds showed some inhibitory power, that nearly all inhibited completely at 1 : 10,000, while many also inhibited at 1 : 20,000.

With only 3 compounds, allyl alcohol mercuric acetate, double salt of methylene blue and mercuric chloride, and 1-amino, 2 (p-naphthalin azo-phenyl mercuric acetate) 5 sulphonic acid, did the bacteriostatic action go farther than 1 : 20,000.

The bactericidal power of the compounds used in these experiments has not been tested, partly because of the insolubility or difficult solubility of most of the preparations. In most of the experiments with antiseptics and drugs used in my former experiments, we have found that a somewhat lower concentration was required for inhibition than for complete bactericidal action. In a report by DeWitt and Sherman,[12] we found that human tubercle bacilli distributed in a thin layer on garnets and exposed for 24 hours to mercuric chloride in a dilution of 1 part of the drug to 100,000 parts of water were killed so that they failed to infect guinea-pigs.

It required 1 part in 50,000 to kill them in clumps in 1 hour so that they would not grow on agar tubes and 1 part in 1,000 of water to kill them in 1 hour so that they would not cause the disease in animals. In all cases, the garnets, after exposure to the mercuric chloride solutions, were well washed with ammonium sulphide solution to neutralize the mercury and then with 4 wash waters, so that none or very little of the drug could have been carried over to continue its action. It is difficult to explain this apparently much higher bactericidal than bacteriostatic power of mercuric chloride except on the assumption that a considerable part of the mercury must have been thrown out of solution in the agar. However, Lewis [13] concludes that there is no close or constant correlation between the bactericidal and inhibitory action of chemicals.

THERAPEUTIC EXPERIMENTS

1. Mercurous Chloride

Six guinea-pigs were inoculated subcutaneously with 0.2 c c of a suspension of human tubercle bacilli of the strain known as "Old Human" to distinguish it from the various strains acquired more recently. The suspension was diluted to the point at which it was just slightly opalescent. These 6 animals were each fed daily one pill containing 1 mg. of mercurous chloride. One pig died in 2 days, before any tuberculous involvement was possible. The others lived

[12] J. Infect. Dis., 1914, 15, p. 245.
[13] J. Exper. Med., 1917, 25, p. 441.

167, 231, 240, 245, and 325 days, an average of 241 days, and all showed marked tuberculous involvement of lymph glands, spleen, liver and lungs. They had received by mouth 125, 198, 209, 213, and 282 mg., an average of 205 mg. Control pigs were inoculated on the same day with the same dose of the same suspension and lived 85, 183, 193, 193, and 193 days, an average of 169 days. All the control animals also showed marked generalized tuberculosis. The only difference, then, between the treated and the untreated animals was that the treated animals lived longer than the untreated, but the extent of the disease at death was about the same.

2. Mercuric Sulphocyanide

Six guinea-pigs were inoculated with 0.2 c c dilute suspension of the strain known as "Old Human." They received one subcutaneous injection of 0.5 mg. and one of 1 mg. and were fed 1 mg. of the drug per day. The compound was toxic enough so that daily feedings had to be interrupted at intervals for a week or more and then resumed. Two of the animals received one intracardiac injection of 1 mg. One of these died the next day and the other 33 days later. The length of life after inoculation was 42, 85, 96, 143 and 193 days. The average length of life was 112 days. The amount of the drug administered was 39.5, 60, 93, 124, 166 mg., or an average per animal of 91.5 mg. All the animals had enlarged caseous inguinal glands. The one dying in 42 days had no other tubercles visible macroscopically. The others showed some tubercles in the organs but only in the one living 193 days was the disease marked.

The duration of life of the untreated controls was 98, 111, 116, 117, 143 and 156 days, or an average of 123.5 days. Hence with this treatment, the treated animals died more quickly than the untreated controls. On the other hand, the extent of the disease in all the controls was much greater than in the treated animals, the liver, spleen and lungs in those dying after 98, 111, 116, and 117 days being more involved than in the treated animals living 143 and 193 days. This suggests that the drug had a certain slight inhibitory action over the progress of the disease although not enough to prevent it completely, while its toxic action was sufficient to shorten life in many of the animals.

3. Mercury Potassium Cyanide

Six guinea-pigs were inoculated with 0.2 c c of dilute emulsion of "Old Human" tubercle bacilli. Each received one subcutaneous, one intracardiac and one intramuscular injection and was fed 1 mg. per day. One died on the eighth day after inoculation with no sign of tuberculosis. One died on the thirty-second day showing an enlarged regional gland and a few small tubercles in the spleen. The others lived 86, 162, 169 and 238 days, or an average of 166 days. All showed marked tuberculosis of lymph glands, spleen, liver and lungs. The controls of this series lived 98, 111, 116, 117, 143 and 156 days, or an average of 123.5 days, and all had a marked generalized tuberculosis. In this series, if we leave out of consideration 2 treated animals that died early from some cause other than tuberculosis, the treated animals lived longer than the controls, but the extent of the disease was about the same in the two sets.

4. Allyl Alcohol Mercuric Acetate

This preparation was made for me in the chemical laboratory of the University of Chicago. My reason for having it made was the reputation that allyl alcohol and some of its derivatives have acquired in the clinical treatment of tuberculosis. As is seen in table 2, this mercurial compound of allyl alcohol has a high bacteriostatic power over the growth of the human tubercle bacillus. The compound could be dissolved in water to make a solution of one part in 1,000. Six guinea-pigs were inoculated with 0.05 mg. of culture strain 1305, isolated by Dr. H. J. Corper at the Chicago Municipal Sanatorium about 5 years ago. Each animal received a subcutaneous injection of 1 mg. of the allyl alcohol mercuric acetate in 1 c c of water. Then each was fed 1 mg. pill per day up to the time of death. These animals lived 46, 56, 99, 102, 104 and 140 days, or an average of 91 days. They had received 41, 49, 87, 87, 88 and 118 mg. of the drug, or an average of 78 mg. for each animal. All showed marked tuberculosis of lymph glands, spleen, liver and lungs. The controls of this series lived 77, 78, 79, 84, 119 and 119 days, or an average of 92.6 days. All the animals showed marked generalized tuberculosis. In spite then, of the high bacteriostatic power of allyl alcohol mercuric acetate in vitro, it seems to have no therapeutic effect on animals inoculated with a virulent strain of tubercle bacilli, since both length of life and extent of disease are practically the same in the treated and untreated animals. It is probable that this drug fails to penetrate the young tubercles, or is broken up and loses its antiseptic power before it reaches them.

5. Mercuric Succinimide

This is the mercury compound which, perhaps because of its being a soluble organic mercury compound, has been especially used in the clinical treatment of tuberculosis. It was used largely by B. L. Wright and his followers, although some of them substituted mercuric chloride for the succinimide. Six guinea-pigs were inoculated with 0.2 c c of the dilute suspension of the "Old Human" strain of tubercle bacilli. Subcutaneous and intracardiac injections were used at first, then pills containing 1 mg. of the drug were fed daily. One lived only 10 days, succumbing on the day following an intracardiac injection and, of course, showed no tuberculosis. The others lived 85, 95, 154, 189 and 219 days, an average of 148 days. They received 84, 90, 154, 214 and 240 mg. of the drug, or an average of 156 mg. The extent of the disease was distinctly limited in this series. The pig that died in 85 days showed only an involvement of the regional lymph glands with necrotic masses in the spleen. Even the one that lived the longest showed but slight involvement of the spleen and lungs and regional lymph glands. Some had partial necrosis of the liver and some showed cirrhosis of the liver, but the involvement of the organs was slight for the duration of the disease. The controls lived 98, 111, 116, 117, 143 and 156 days, or an average of 120 days. All had marked tuberculosis of lymph glands, spleen, liver and lungs. The treated animals have lived on the average somewhat longer than the controls, although 2 died earlier and 2 lived much longer than any of the controls. The extent of the disease in the treated was considerably less than in the untreated animals.

6. Mercuric Salicylate

Five guinea-pigs were inoculated with 0.2 c c of dilute suspension of Miller's strain of human tubercle bacilli, a moderately virulent strain. Since mercuric salicylate is insoluble, a suspension was made in sterile paraffin oil and an amount containing 2 or 3 mg. of the drug injected intramuscularly once a week. On the other days of the week, a pill, containing at first 1 mg. and afterward 2 mg., was fed to each animal. This dosage was evidently too toxic, as the animals lived only 13, 27, 27, 37 and 59 days, having received 12, 24, 24, 35 and 67 mg. of the drug. The inguinal glands were enlarged and caseous in all except the one dying in 13 days, and the spleen was involved in some of the others. Hence, even in these doses which so quickly killed the animals, there was evident little, if any, influence on the progress of the infection. The controls of this series lived 91, 96, 97, 104, 121 and 226 days, an average of 122.5 days, and all showed marked generalized tuberculosis.

7. Mercuro-Iodo-Hemol

This is an insoluble commercial preparation obtained from Merck and Company. Hemol is a hemoglobin preparation, and the compound used is said to contain 12.35% of mercury and 28.6% of iodine. From 2-5 grains by mouth three times per day is the dose recommended for syphilitic patients. Six guinea-pigs were inoculated with 0.2 c c of a dilute suspension of the "Old Human" strain of tubercle bacilli. Each animal was fed daily a pill containing 1 mg. of the mercuro-iodo-hemol preparation and received several subcutaneous injections of a suspension in gelatin and an intramuscular injection. They lived 67, 96, 110, 133, 135 and 171 days, or an average of 120 days, and received 41, 86, 97, 109, 119 and 147 mg. of the drug, an average of 99% mg. All showed considerable tuberculous involvement of the lymph glands, liver, spleen and lungs. The controls of this series lived 98, 111, 116, 117, 143 and 156 days, or an average of 123.5 days, and all showed an extreme degree of generalized tuberculosis. Hence, the hemol mercury preparation, while not especially toxic, seems to have had no beneficial influence on the progress of the disease.

8. Mercurol

Mercurol or mercury nucleide is also a commercial preparation obtained from Merck and Company. It is soluble in water and is said to contain 10% of mercury. It was tested in this series of experiments, especially because of the favorable reputation which nuclein and nucleinic acid have long borne in the clinical treatment of pulmonary tuberculosis. The mercurol is freely soluble and neither very toxic nor locally irritating, so that injections of various kinds could be continued much longer than with most of the mercurial preparations. Several sets of animals were therefore tried out in testing the therapeutic properties of this drug.

(a) Six guinea-pigs were inoculated with 0.2 c c of a dilute suspension of "Old Human" tubercle bacilli. These were fed daily pills containing each 1 mg. of the drug, the daily dosage being increased according to the condition of the animal. They also received injections once a week, 2 subcutaneous injections of 1 and 3 mg. and 4 intracardiac injections of 2, 3, 4 and 5 mg. of the drug. They lived 33, 42, 47, 56, 82 and 234 days, or an average of 82 days. They received 36, 49, 57, 76, 129 and 349 mg., or an average of 116 mg. of the drug. Three of these animals died as a direct result of an intracardiac injection, one died from a septic abortion, while none had

any considerable tuberculous involvement more than enlarged and partly caseous regional lymph glands. The controls of this set lived 26, 56, 71, 137, 202 and 217 days, or an average of 118 days, and all except the first two to die had marked generalized tuberculosis.

(b) The second set consisted of 6 guinea-pigs inoculated with the same dose of the same strain as set 1. They were treated merely by feeding mercurol pills, gradually increasing the dose as the condition of the animal permitted. Two other guinea-pigs were inoculated with the caseous substance from a lymph gland taken from a pig of set 1 and were treated with the pigs of set 2, after a local tubercle developed. These lived 48, 90, 122, 139, 160, 271, 271, 271 days, or an average of 171 days. They received by mouth 42, 86, 166, 199, 186, 186, and 186 mg. In all these animals there were found enlarged caseous glands in the inguinal region and from slight to moderate involvement of the liver, spleen and lungs. In none of the animals of this set were these organs normal or even completely free from tubercles, as they were in set 1. The controls of this set lived 85, 183, 193, 193 and 193 days, or an average of 169 days, a slightly lower average length of life than in the treated animals because 3 of the treated animals lived much longer than the others and much longer than any of the controls. All of the controls, except the one dying on the eighty-fifth day, exhibited much more marked general tuberculosis than did the treated animals.

(c) The third set was inoculated with the same dose of the same strain as the other two sets. It was treated by feeding and intraperitoneal injections. These died in 47, 78, 118, 159 and 164 days, or an average of 113 days. They had received 52, 78, 126, 142 and 155 mg., or an average of 111 mg. of the drug. The one dying after 47 days had enlarged caseous glands in both groins and a few in the spleen and liver. The one dying in 78 days had no caseous glands and no macroscopic tubercles in the liver, spleen or lungs. The 3d, 4th and 5th to die showed no tubercles in any of the organs and no caseous glands. This pathologic condition would have seemed somewhat encouraging for the claims of mercurol had it not been that the control animals of that set lived much longer than the treated and exhibited about the same degree of tuberculous involvement. This seemed to indicate that the "Old Human" strain was losing its virulence to a degree which made it hardly usable for therapeutic experiments. In connection with set 3, a number of uninoculated guinea-pigs were treated with mercurol in the same way as the infected pigs. These pigs were killed after the tuberculous pigs had died. No pathologic changes were noted, except hemorrhages and hemorrhagic exudates in most of the animals.

In regard to mercurol, we must conclude that, while it is quite nontoxic and nonirritating and seems to have some slight inhibiting influence over the spread of the disease in the animal, that influence is not sufficient to encourage us in further experiments, since in all probability, with more virulent strains, the influence of the drug would either not be felt or, as often happens, would stimulate the organism to increased growth and virulence.

9. Mercury Phenolphthalein

This preparation was made for me in the chemical laboratory of the University of Chicago according to suggestions made by Hahn and Kostenbader.[14] It is insoluble in water but dissolves in alkalies. The mercury makes up about 39% of the molecular weight. (See table 1.)

[14] Ztschr. f. Chemotherapie, O., 2, O, p. 71.

Six guinea-pigs were inoculated with 0.2 c c of a dilute suspension of "Old Human" strain of tubercle bacilli and each was fed daily first one and later two pills containing each 2.6 mg. Three of the 6 animals died during the first month of some cage infection with no sign of tuberculosis. The other three lived 210, 244 and 306 days and, at death, showed advanced tuberculous involvement of lymph glands, liver, spleen and lungs. The controls died after 126, 170, 182, 187, 192 and 205 days, all showing marked general tuberculosis.

10. Mercury Tetraiod Phenolphthalein

This was also suggested by Hahn and Kostenbader and, having 4 iodin atoms in the molecule given for mercury phenolphthalein, was also tested, the animals being inoculated and then fed daily. Aside from 2 animals that died early, they lived 133, 151, 166 and 241 days, and all showed at death advanced tuberculosis of lymph glands, spleen, liver and lungs. The controls lived 122, 133, 150, 169, 176 and 295 days. All had extensive advanced tuberculosis. Hence these 2 phenolphthalein dyes seem to have no influence on the extent and distribution of the disease.

11. Double Salt of Fluorescein and Mercuric Chloride

This also is one of Hahn and Kostenbader's "mercurialized dyes." Six guinea-pigs were inoculated with 0.2 c c of a dilute suspension of the "Old Human" strain and then fed with the dye. Three died during the first month of cage infections. The other three lived 58, 88 and 217 days having received 250, 426 and 950 mg. of the dye. The one living 58 days showed no generalized tuberculosis, the second contained tubercles only in the lungs, while the third had only a few small tubercles in the lungs, but the liver and spleen were full of tubercles. The controls lived 126, 170, 182, 187, 192 and 205 days, or an average of 177 days, and all showed extensive advanced general tuberculosis.

In spite of the fact that the involvement in the controls was much greater than in the treated pigs, we cannot under these conditions consider that the difference was due to the treatment, since all animals that lived as long as did the controls had some generalized tuberculosis, even though not distributed through all the organs.

12. Double Salts of Trypan Blue and Mercurous Chloride and Mercuric Chloride

These salts were made for me by Dr. Walter Fraenkel, and the method of making was described in a previous paper.[15] At that time it was found that these salts had some tuberculocidal power since 0.1 c c of a suspension of tubercle bacilli exposed for 24 hours to 1% solution of the salts and then injected into guinea-pigs failed in most cases to cause disease. The inhibiting action of the mercuric salt was surprisingly low, since only the 1 : 1,000 and 1 : 5,000 dilutions caused complete inhibition. Six guinea-pigs were inoculated with 0.2 c c of dilute suspension of the "Old Human" strain. They received the mercurous salt of the dye both by daily feeding of 1, 2 or 3 mg. and by occasional intramuscular injections of a suspension in oil. They lived 33, 35, 38, 73, 93 and 219 days, or an average of 81 days. They had received

[15] Jour. Infect. Dis., 1914, 14, p. 498.

37, 43, 52, 97, 181 and 192 mg., or an average of 100 mg. All showed generalized tuberculosis in moderate degree. A second set was inoculated with 0.1 c c of the Miller's, a more virulent strain. These were both fed and injected. They lived 91, 94, 137 and 139 days, or an average of 115 days. They received 110, 112, 137 and 139 mg. of the dye. All showed an advanced generalized tuberculosis. Hence, these mercury salts of trypan blue have only slight bactericidal and inhibitory power and no therapeutic influence over the disease.

13. Mercury Derivations of Aromatic Amines

Jacobs and Heidelberger[16] described some mercury derivatives of aromatic amines. As some of these preparations looked as if they might be of interest in my work, I had my chemist make two of them according to their directions.

(a) 4-ortho-oxybenzylidene amino phenyl para mercuric acetate, which contains 44.02% of mercury. This compound is soluble in dilute alkali. Six guinea-pigs were inoculated with 0.2 c c of a dilute suspension of "Old Human" strain of tubercle bacilli. One mg. pills were fed to each pig daily and several injections were given. Two of the pigs died shortly after an intracardiac injection. The other animals lived 33, 68, 78 and 220 days, or an average of 99¾ days. They had received 32, 40, 53 and 151 mg., or an average of 69 mg. per animal. The three that died earliest had no or slight tuberculosis, but the one living 220 days showed an extensive generalized tuberculosis. The controls lived 24, 119, 184, 238, 264 and 315 days, or an average of 207 days, much longer than the treated animals and all except the first showed marked and extensive generalized tuberculosis.

(b) 1-amino-2(para-naphthalin azophenyl mercuric acetate) 5-sulphonic acid. This contained 34.24% of mercury and was also soluble in dilute alkali. The inhibitory power was high, since there was complete inhibition up to 1:20,000 and partial inhibition even to 1:500,000. Two sets of animals were used for this compound. The first set was inoculated and treated in the same way as the set just described. These lived 10, 30, 50, 51, 65 and 250 days. The first ones died from the effect of an injection. Only the one that died last after 250 days showed tuberculosis. The second set was inoculated with the same dose of the same strain and treated mostly by feeding, only one subcutaneous injection having been given. These animals lived 36, 118, 146, 155, 188 and 255 days, an average of 149 days. All showed slight to moderate generalized tuberculosis with considerable tendency to the fibrous form of tuberculosis. The controls lived 42, 168, 199, 219, 276 and 281 days, or an average of 197 days, much longer than the treated pigs, and all except the first showed an advanced generalized tuberculosis.

14. Mercury Compounds of Methylene Blue and Its Derivatives

This group of compounds has been of especial interest to me because of the position methylene blue itself has occupied as an internal germicide and also in the treatment of tuberculosis. Some of the work has already been reported briefly.[17] In this present report, the work of the various mercuric compounds of the group of dyes related to and derived from methylene blue will be reported more in detail. In the earlier work and by different methods than those

[16] J. Biol. Chem., 1914, 20, p. 513.

[17] DeWitt: Jour. Infect. Dis., 1913, 13, p. 178; Transactions of the Twelfth Annual Meeting of the National Tuberculosis Association, 1916.

used, I found the methylene blue chloride caused complete inhibition of the human tubercle bacillus in a dilution of 1:5,000, while Paul Lewis[18] by still another method found that 1:1,000,000 was the highest concentration at which the growth equaled that on control flasks. Methylene blue was also shown by me to have some bactericidal action on the tubercle bacillus.

(a) The double salt of methylene blue and mercuric chloride, which was made by my chemical assistants by adding a solution of mercuric chloride to a solution of methylene blue and thoroughly washing and drying the precipitate thus formed, contains about 34% mercury and is quite insoluble, but forms a good suspension on shaking in hot agar and then cooling and slanting quickly. There was no growth in tubes up to 1:20,000 and only slight growth in the tubes containing a dilution of 1:50,000. The amount of mercury per c c in the highest dilution which completely inhibited was 0.014, which was less than completely inhibited in any other mercurial tested, except the allyl alcohol mercuric acetate. In the first experiment with this drug, 4 of the 6 guinea-pigs died within the first 3 weeks. The other 2 lived 130 and 208 days. Each pig was fed 1-2 mg. every day during life and received injections once a week. There was practically no tuberculous involvement even in the animal that lived 208 days. Another set of animals was therefore inoculated in the same way with 0.2 c c of dilute suspension of the "Old Human" strain of tubercle bacilli. These received no injections but were fed daily pills containing a dose of 1-2 mg. These pigs lived 147, 237, 265, 269 and 288 days, an average of 241 days. They received by mouth during life 264.6, 460, 460, 500 and 520 mg. of the drug, or an average of 440.9 mg. per animal. Four of these pigs showed at necropsy only slight involvement of regional lymph glands and of the spleen; but the fifth showed extensive and advanced tuberculosis of lymph glands, spleen, liver and lungs. The controls of this series lived 126, 170, 182, 187, 192 and 205 days, an average of 177 days, a much shorter time than the treated animals lived, while every one of the controls showed marked, extensive and advanced tuberculosis of lymph glands, spleen, liver and lungs. These results were so good that it seemed well worth while to carry the experiment further, and another set of guinea-pigs was inoculated with the same dose of the same strain of human tubercle bacilli. These also were fed daily and no injections were given. These lived 96, 188, 247, 267, 298 and 298 days, an average of 231 days. In 4 of these the tuberculous involvement was slight, while in 2 it was extensive. The controls of this series lived 42, 168, 199, 219, 276 and 281 days, an average of 197 days, and all except the first showed as extensive tuberculosis as the worst two of the treated animals. The effect of treatment in this set seemed much less than in the other sets described, but the treated animals lived longer and showed less extensive disease than the untreated.

The next set consisted of 5 guinea-pigs inoculated with 0.2 c c dilute suspension of the "Old Human" strain. They received the drug by daily feeding of 1-2 mg. pills and intramuscular injection of the drug in cotton seed oil. They lived 100, 147, 265, 335 and 733 days. None of these animals had any definite tubercles in any of the internal organs, though all had slightly enlarged noncaseous glands in the inguinal region. The controls of this set lived 145, 172, 222, 232 and 266 days, an average of 207 days, and all had advanced tuberculosis of lymph glands, liver, spleen and lungs. The next set consisted

[18] Jour. Exper. Med., 1917, 25, p. 441.

of 6 guinea-pigs inoculated with the same dose of the same strain as the other sets and treated by daily feedings and weekly intramuscular injections of the double salt in cotton seed oil. They lived 97, 101, 103, 161, 402, 813 and 1,000 days. The 2 that lived over 2 years showed no sign of tuberculosis except slightly enlarged and hard glands in the groins, which showed necrotic fibrous encapsulated tubercles. The others showed no sign of tuberculosis, except some enlarged, noncaseous glands. This would have been satisfactory had it not been that the controls of this set, although they died much earlier than some of the treated animals, living 110, 160, 204, 233 and 234 days, and although they had enlarged and caseous glands, showed but slight or moderate tuberculosis of the internal organs. It seemed safe to conclude from all these experiments that animals inoculated with a strain of low virulence certainly received considerable benefit from treatment with small doses of the double salt of methylene blue and mercuric chloride. A much more virulent strain was then used to determine whether the effect observed in the animals inoculated with the strain of low virulence would also be observed in those inoculated with a strain of high virulence. The strain used was called "1305" and was one isolated in about 1916 by Dr. Corper from sputum obtained from a patient in the Chicago Municipal Tuberculosis Sanatorium. Eight guinea-pigs were inoculated subcutaneously, each receiving 0.05 mg. of the culture. They were treated by daily feeding of pills containing each 1 mg. of the double salt of methylene blue and mercuric chloride. They lived 54, 58, 70, 76, 77, 111, 121 and 122 days, or an average of 73 days. They had received 42, 50, 60, 65, 66, 98, 104 and 105 mg. of the drug, or an average of 74 mg. per animal. All the animals showed some tuberculosis of the internal organs at necropsy and in nearly all the disease was moderately or extremely extensive. The controls lived 60, 109, 111, 129 and 172 days, or an average of 116 days, and all showed marked tuberculosis of the lymph glands, spleen, liver and lungs. In this set there was practically no difference between the treated and untreated guinea-pigs. Another set was inoculated with 0.05 mg. of 1305, then fed 1 mg. of the double salt daily, as in the last set, but these also received several subcutaneous injections of 1 mg. of egg albumin. These lived 92, 104, 106, 121, 124 and 214 days, and nearly all showed extensive involvement of lymph glands, liver, spleen and lungs. Still another set, inoculated in the same way with the same dose of the same strain as the set described above and as the controls given under the first of these three 1305 sets, received in addition to the feeding with the double salt of methylene blue and mercuric chloride several subcutaneous injections of a vaccine made by heating a suspension of the same strain with which they were inoculated to 60 C. for one hour. These lived 30, 67, 85, 111, 130, 143, 143, 153 and 156 days, or an average of 113 days as compared with an average of 116 days life of the controls. All of the guinea-pigs of this set also showed extensive tuberculosis of the internal organs in a degree that is apparently about equal to that of the control animals.

Another set was inoculated each with 0.05 mg. of the virulent strain 1305 and then fed daily from 1 to 2 mg. of the double salt. They lived 62, 73, 95, 96, 101, 129, 164 and 259 days. They had received by mouth before death 63, 73, 92, 93, 97, 121, 151 and 389 mg. Five of these 8 guinea-pigs showed either no or slight tuberculous involvement, having died from some acute infection. The 2 that died after 96, 101 and 259 days showed a rather extensive general involvement. Six of the 8 controls of this set have died after

14, 66, 66, 207, 213 and 267 days. The one dying after 14 days showed no tuberculosis, and one of those dying after 66 days had a slight degree of the disease; in the other 4 the glands, liver, spleen and lungs were extensively involved.

Hence, we must say that, as far as we may conclude from these experiments, the double salt of methylene blue and mercuric chloride, if fed in 1 mg. doses, exerts no or but little beneficial influence on the rapidly progressive type of tuberculosis caused in guinea-pigs by inoculation with a virulent human strain, although in the type caused by inoculation with a strain of low virulence, it seems to exert a distinct and considerable influence, although it cannot be said to cure, even in these cases. If we may judge by the length of life and by the condition of the animals, we cannot consider that it had in any case an injurious influence if given by mouth and, if given by intramuscular injection, there seems to have been no other ill effect than the local infiltrations and occasional ulcerations usually caused by any oil emulsion.

In addition to the experiments described on the use of the double salt of methylene blue and mercuric chloride, 2 sets of animals were treated with alternate injections of methylene blue and of calomel and one set using alternate injections of methylene blue and of mercuric chloride. This was done with the idea that a compound of methylene blue and mercury might thus be formed internally and perhaps be more efficacious. The first set treated with methylene blue and calomel died during the first month, showing no sign of tuberculosis. The second set received the methylene blue by intracardiac injection and the mercurous chloride by intramuscular injection, but both in smaller individual doses than were used in the first set of animals. Three of the animals of this set died after the first intracardiac injection, 2 from abortion and the others from the heart injection. The other 3 received the injections about once a week for one month and afterward no further treatment and lived 172, 211 and 312 days. Only the last one of these showed any considerable tuberculous involvement. The controls of this set lived 26, 56, 71, 137, 202 and 217 days. The strain used for inoculation in this set was the "Old Human" which had by this time partly lost its virulence, so that only the controls that had lived 200 or more days showed extensive tuberculosis, while in the treated animals, only the one that lived 312 days showed any considerable disease.

The next set were inoculated subcutaneously with 0.2 c c of "Old Human" strain of tubercle bacilli and for a little over one month received weekly intraperitoneal injections of methylene blue and mercuric chloride separately. These drugs were also fed to them on alternate days. They lived 41, 65, 106, 106, 110 and 247 days, or an average of 113 days. Those that lived 106 and 110 days showed moderate generalized tuberculosis, but the others, even the one living 247 days, showed none. The controls of this set lived 110, 160, 204, and 233 and 234 days and showed little generalized tuberculosis. All showed enlarged and partly caseous glands. Hence this treatment of methylene blue and mercuric chloride given separately cannot be said to have had any beneficial effect even in the animals inoculated with a strain of human tubercle bacilli of extremely low virulence.

(b) Double salt of iod methylene and mercuric chloride. Iod methylene blue was made by my chemical assistant and was found to inhibit growth of tubercle bacilli in dilutions of 1 : 5,000, just as did methylene blue itself. It

is probably the same compound recommended by von Linden. The double salt was made by adding a solution of mercuric chloride to a solution of iod-methylene blue and washing the precipitate thus formed. It contains 24¾% of mercury and its inhibiting power was a little less than that of the double salt of methylene blue and mercury, since there was slight growth in the tubes containing the dye in a dilution of 1 : 20,000, about corresponding to the growth in the tubes containing dilutions of 1 : 50,000 of methylene blue and mercuric chloride. Six guinea-pigs were inoculated with 0.2 c c of a dilute suspension of "Old Human" strain of tubercle bacilli and then fed the dye daily in 1 mg. doses and injected once a week subcutaneously during the first month. They lived 81, 142, 143, 165, 204 and 453 days, an average of 198 days, and had received 89, 156, 157, 178, 224 and 509 mg. of the dye, or an average of 219 mg. per animal. All the animals of this set exhibited extensive tuberculosis. The controls lived 122, 147, 176, 197, 215 and 238 days, or an average of 182.5 days. The average duration of life of the controls was less than that of the treated animals, but that was due to the one animal of the treated set which lived more than twice as long as any of the others and almost twice as long as the last of the controls to die. The extent of the disease in the controls was about the same as in the treated animals, so that the double salt of iod methylene blue and mercuric chloride seems to have had in this experiment no beneficial action, although the strain with which the animals were inoculated was one of fairly low virulence. This may partly confirm a statement made by Lenard[19] that iodin prevents the deposition of the mercury in the liver, which is said by Blumenthal and Oppenheim[20] to have something to do with the therapeutic power.

(c) Double salt of methylene green and mercuric chloride. Methylene green has one nitro group in the methylene blue molecule. The dye itself has not in my experiments shown itself equal to the methylene blue in inhibitory, bactericidal or therapeutic power. The mercury preparation has been made by my assistants by the same method as the other double salts. It is quite insoluble. Six guinea-pigs inoculated with 0.2 c c of a dilute suspension of "Old Human" strain were fed daily and received several injections. Four of the 6 died within the first month, the other 2 living 201 and 238 days and showing extensive and advanced tuberculosis. Therefore another set was inoculated in the same way and received no injections, but simply daily feedings. These lived 73, 199, 220, 226 and 378 days, an average of 219 days. They had received 114, 398, 413, 450 and 648 mg., or an average of 405 mg. of the double salt. The first of these animals that died showed no tuberculosis except a small gland and a few tubercles in the spleen. The third also showed but a slight degree of tuberculosis. The second and fourth had a moderately advanced tuberculosis and the last exhibited signs of healed and healing tubercles, as the groins contained small calcified granules and in the lungs and liver the tubercles were hard and fibrous. The controls of this set lived 126, 170, 182, 187, 192 and 205 days, or an average of 177 days. All the controls showed extensive, advanced tuberculosis. If we consider in this experiment the consistently longer life of the treated animals, the less extent of the disease, and the tendency to healing shown in the guinea-pig that lived longest, we may conclude that the double salt of methylene green and mer-

[19] Ztschr. f. Chemotherapie, 1914, 2, p. 106.
[20] Biochem. Ztschr., 1914, 65, p. 460.

curic chloride, as well as that of methylene blue and mercuric chloride, shows some beneficial influence on tuberculosis caused by a strain of tubercle bacilli of relatively low virulence. The strain of high virulence was not tested with this compound.

(d) Diazo amino methylene blue ortho toluidin dimercuric acetate was made for me by my chemical assistant, Doctor Kharasch. The method of making this compound and the two following ones will be reported by him later. The chemical formula for it is given in table 1; it contains 41.3% of mercury, and is quite insoluble. Its inhibitory and germicidal power have not yet been determined. Seven guinea-pigs were inoculated subcutaneously, each with 0.05 mg. of the virulent strain 1305. They were fed daily and received one subcutaneous injection. They lived 8, 32, 100, 106, 137, 140 and 148 days, an average of 96 days, and received 7, 29, 87, 92, 119, 121 and 128 mg. of the compound. All except the 2 that died after 8 and 32 days showed a high degree of advanced generalized tuberculosis. The controls of this set lived 51, 83, 83, 94, 127 and 143 days, or an average of 97 days, and all showed marked and extensive tuberculosis of lymph glands, spleen, liver and lungs. This experiment, therefore, would not indicate that this compound of methylene blue has any beneficial action on this virulent tuberculous infection.

(e) Diazo amino methylene blue brom hydrate ortho toluidene di mercuric acetate. The formula for this is given in table 1, and it contains 39.5% of mercury. It is insoluble. Nine guinea-pigs were inoculated subcutaneously with 0.05 mg. of strain 1305 and then given one injection of the compound suspended in oil and a daily feeding of 1 mg. They lived 8, 23, 32, 76, 81 95, 129, 133 and 170 days, or an average of 92 days, if we omit the accidental death at 8 days. They had received 10, 21, 64, 69, 81, 131, 125 and 145 mg., or an average of 78 mg. per animal. All except the 3 animals dying after 8, 23, and 32 days, had a pronounced general tuberculosis. Hence this compound, like the same compound without the brom hydrate, has no beneficial influence in this virulent infection.

(f) Diazo amino methylene blue ortho phenol mercuric chloride contains 29.7% of mercury. Ten guinea-pigs were inoculated with 0.05 mg. of strain 1305 and were then fed daily 1 mg. and received one injection of the compound suspended in oil. These lived 11, 16, 18, 48, 72, 126, 144, 154, 171 and 174 days or an average of 127 days, after omitting the first two. They received 14, 19, 21, 35, 55, 101, 117, 125, 126 and 129 mg. of the compound, an average of 74.2 mg. The first 2 died from septic abortion and the third from acute pneumonia. Four of the others showed generalized tuberculosis of moderate degree, while the other 3, which were the last to die, exhibited a slight degree of tuberculosis, a few small tubercles in one of the organs or perhaps only an enlarged gland. The controls of e and f lived 62, 125, 125, 128 and 138 days, or an average of 115 days, and all showed advanced generalized tuberculosis.

Concerning the 3 diazo compounds of methylene blue and mercury just described, but little can be said. A number of the treated animals died early, suggesting a toxic and weakening action of the drug. With the 2 o-toluidin compounds d and e, there was no difference between the treated and untreated animals either in length of life or in extent of the disease. The animals treated with the o-phenol compound f lived slightly longer and had less tuberculous involvement than the controls, although they were inoculated with the virulent strain 1305.

The results of the chemotherapeutic experiments with these 24 mercuric compounds have been described as briefly as possible in the foregoing pages. But, in order to compare these results, a brief summary is given in table 3. It will be seen from this table that, as compared with the untreated controls that had been inoculated with the same dose of the same strain of tubercle bacilli, several showed a greater duration of life and less extent of disease. In nearly all of these, the infection was due to a strain of lower virulence, and when the same drug was tested with a strain of high virulence no effect was noticed.

TABLE 3

SUMMARY OF RESULTS

Mercury Compounds	Strain of Tubercle Bacilli	Length of Life Compared With Controls	Extent of Disease Compared With Controls
Mercurous chloride	O. H.	Greater	Same
Mercury sulpho cyanide	O. H.	Slightly less	Less
Mercury potassium cyanide	O. H.	Sl. greater	Same
Allyl alcohol mercuric acetate	1305	Same	Same
Mercuric succinimide	O. H.	Greater	Less
Mercuric salicylate	M.	Less	Same
Mercuro-iodo-hemol	O. H.	Slightly less	Same
Mercurol (Merck)	O. H.	Less	Slightly less
Mercury phenolphthalein	O. H.	Greater	Same
Mercury tetraiod phenolphthalein	O. H.	Same	Same
Fluorescein and mercuric chloride	O. H.	Less	Same
Trypan blue mercurous chloride	O. H.	Same	Same
Trypan blue mercuric chloride	O. H.	Same	Same
4-ortho-oxybenzylidene amino phenyl para mercuric acetate	O. H	Less	Same
1-amino-2(p-naphthalin azo phenyl mercuric acetate) 5-sulphonic acid	O. H.	Less	Less
Methylene blue and mercuric chloride	O. H.	Much greater	Much less
	1305	Same	Same or less
Iod methylene blue and mercuric chloride	O. H.	Same	Same
Methylene green and mercuric chloride	O. H.	Greater	Less
Diazo-amino methylene blue o-toluidin mercuric acetate	1305	Same	Same
Diazo amino methylene blue from hydrate o-toliudin mercuric acetate	1305	Same	Same
Diazo amino methylene blue o-phenol mercuric chloride	1305	Greater	Less

Only with one set of animals treated with the double salt of methylene blue and mercuric chloride and with a set treated with diazo amino methylene blue o-phenol mercuric chloride has there been a similar beneficial effect on animals inoculated with a virulent strain, and with none of these drugs has the effect been sufficient so that we could speak of having really cured the disease. As is well known, the guinea-pig is far more susceptible to tuberculosis than is the human being and responds to infection with a more rapidly progressive and fatal disease than does man. Hence a treatment that would cure a tuber-

culous guinea-pig would presumably cure man more easily. A drug that has a decidedly beneficial effect in the guinea-pig would probably have a much greater effect in man. It may well be that the disease caused in guinea-pigs by a less virulent strain like the one described in this paper as the "Old Human" resembles far more nearly the disease in man than does the infection with the virulent strains (Miller's or 1305). However that may be, slight as seem the results reported in this paper, they seem sufficient to justify future work with mercurial compounds in the chemotherapy of experimental tuberculosis. Such work with many new organic compounds of mercury is already under way and will be reported at intervals, as results are obtained that seem worthy of presentation.

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MERCURI-ORGANIC DERIVATIVES. THE MERCURIZATION OF AROMATIC AMINES AND ITS RELATION TO THE THEORY OF SUBSTITUTION.

By Morris S. Kharasch[1] and Isadore M. Jacobsohn.[2]

Received February 15, 1921.

The principles underlying the mercurization of aromatic compounds have occupied the attention of one of us (Kh.) for the past two years. The important fact that, in a mono-substituted benzene derivative, the mercury enters *para* or *ortho* to the group already present in the molecule, was pointed out early by Dimroth,[3] and has been substantiated by the vast amount of experimental work done by others. This fact of *ortho* and *para* substitution is particularly striking when one attempts to apply the theories of substitution to mercurization since mercury never enters *meta* to the group already present in the molecule. Even where the usual rules of substitution require that a *meta* derivative be formed, *e. g.*, with benzoic acid, benzophenone, and nitrobenzene, only *ortho* substituted mercury compounds have been isolated.

The phenomenon of mercurization was considered by Dimroth to be similar to that of nitration, sulfonation, etc., in the sense that the position taken by the entering mercury in compounds containing groups now considered electro-negative, *e. g.*, amino or hydroxyl groups, was quite in agreement with the rule of Crum Brown and Gibson. He considered the exceptional behavior of nitrobenzene, benzoic acid, and benzophenone as anomalous reactions which needed to be accounted for. However, until recently, no effort has been made to explain the orientation of mercury in the benzene molecule.[4] The rule of Crum Brown and Gibson, being purely empirical, offers no suggestion as to the mechanism of the

[1] National Research Fellow in Organic Chemistry.

[2] The material presented here is used by I. Jacobsohn in his dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Chicago.

[3] Dimroth *Ber.*, **35**, 2853 (1902).

[4] Schoeller, Schrauth, and Liese (*Ber.*, **52B**, 1777 (1919) apply the theory of Armstrong for substitution in aromatic amines to mercurization, the limitations of which theory will be pointed out later.

reaction, nor does it help to predict whether a compound could be mercurized or not. Therefore, it appeared desirable to apply some of the modern theories of substitution to the orientation of mercury in the benzene molecule.

In the present paper, the authors will confine themselves to the mechanism of mercurization of aromatic amines and also attempt to show that the mechanism suggested for mercurization may be extended to explain satisfactorily orientation of other groups in the case of aromatic amines. It will be shown further, in a condensed critical survey of the theories of others, that none of these is completely satisfactory. For example, most of them fail to account for the fact that dimethylaniline is mercurized readily.

The theory which the present authors have been led to adopt as a result of their extended experimental work, briefly stated, is as follows. The mercurization of an aromatic amine proceeds in two stages: first, the formation of an ammonium salt with the mercuric acetate or chloride to give a compound of the general type I.



This compound is then rearranged to the more stable configuration having the mercury radical attached to carbon, (Type II), the *para* position being usually favored.[5] If this position is already occupied, the mercury goes to the *ortho* position. Furthermore, it is only the ammonium salt that undergoes rearrangment,[6] the instability of the salt being due to the presence of an electropositive group or atom on the amino nitrogen.[7]

This, it will be noted, is the application to mercurization of the theories of substitution of Armstrong,[8] Bamberger,[9] Chattaway[10] and others, and

[5] The fact of rearrangement may be due to a smaller energy content on the part of the rearranged compound. This relationship was shown to hold by Rother and Stoermer (*Ber.*, **46,** 260 (1913)) for a number of compounds, *e. g.*, cinnamic acid and the isomeric allocinnamic acid.

[6] Acree and Johnson, *Am. Chem. J.*, **38,** 265 (1905), have demonstrated that, in the rearrangement of N-chloro-acetanilide, it is the salt and not the free base that rearranges. They fail, however, to attach any importance to the positive charge on the rearranging group, a fact which has already been pointed out by Stieglitz and Stagner, THIS JOURNAL, **38,** 2057 (1916).

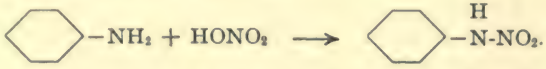
[7] Acree and Johnson, *Am. Chem. J.*, **38,** 265 (1905), have shown that, when N-chloro acetanilide is treated with hydrogen bromide, the speed of the rearrangement is a thousand times greater than when hydrogen chloride is used, and that the end product of the rearrangement is *p*-bromo-acetanilide, instead of *p*-chloro-acetanilide.

[8] Armstrong, *J. Chem. Soc.*, **51,** 258, 583 (1887).

[9] Bamberger, *Ber.*, **27,** 586 (1894).

[10] Chattaway, *J. Chem. Soc.*, **75,** 1048 (1899).

of the general theory of catalysis advanced by Stieglitz,[11] Acree[12] and others that acid catalysts act through salt formation.[13] However, the theory advanced by the present authors diverges from that of Armstrong, Bamberger, and Chattaway, and also from recent theory of mercurization of Schoeller, Schrauth and Liese[4] in a few very important aspects. The theories advanced by the above mentioned authors assume that the entrance of a group into the nucleus of an aromatic amine is always preceded by substitution on the amino nitrogen, thus,



However, as an explanation, this is not adequate to account for the mechanism of substitution of tertiary amines, of the type of dimethyl aniline which contains no replaceable hydrogen. Furthermore, they do not emphasize the nature and importance of the catalysts used in substitution, *i. e.*, strong acids. That the rôle played by acid catalysts is that of salt formation has been pointed out very clearly by Stieglitz[11] in his work on the catalysis of imido-esters, as well as by Acree.[12]

We find thus, that the previous hypotheses have to be modified, if they are to explain consistently all the facts of substitution without further assumptions. The hypothesis advanced by the writers to explain the mercurization of aromatic amines lends itself admirably to this purpose. We shall now proceed to illustrate as briefly as possible the basis for this hypothesis and then show that it is quite compatible with the *ortho* and *para* orientation of other groups in the case of aromatic amines.

That mercury has a great affinity for amino nitrogen and for carbon is best illustrated by the readiness with which the mercury-ammonium complexes are formed, and by the great stability of mercury-carbon compounds.[14] It is the combination of this property of mercury to form stable nitrogen and carbon compounds, and the relatively greater stability of the latter, which enables us to attach mercury to the carbon of the benzene ring in the case of aromatic amines.

Within the scope of a series of investigations being carried out by the one of us, work was undertaken upon the mercurization of the N-alkylated

[11] Stieglitz, *Proc. Congress of Arts and Sci.* (St.Louis), **IV**, 278(1904); *Am. Chem. J.*, **30**, 29 (1908).

[12] Acree, *ibid.*, **37**, 410 (1907).

[13] In this connection, the work of Reilly and Hickinbotham (*J. Chem. Soc.*, **117**, 103 (1919)) on the rearrangement of *n*-butylaniline is quite significant. They found that only those salts which are capable of combining with the amino group effected the rearrangement.

[14] The stability of the mercury-carbon union is a relative term. It depends to a large extent upon the position of the mercury in the molecule and upon the nature of the neighboring groups. This will be discussed in a later paper. See also Stieglitz, Kharasch, and Hanke, THIS JOURNAL, **43**, 1186 (1921).

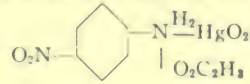
nitro-anilines. It was found that mercurization does not take place when *p*-nitro-dimethylaniline is treated with mercuric acetate in alcoholic solution. Though various experimental conditions were tried, the efforts were futile. It seemed, however, in view of the results of an investigation carried out with other nitro-anilines,[15] that mercurization should take place unless there was some property of the mercurizable nitro-anilines which was not shared by the *p*-nitro-dimethylaniline. Reference to the literature at once revealed the fact that *p*-nitro-dimethylaniline is an extraordinarily weak base. It is precipitated from even conc. hydrochloric acid solution as the free base instead of its hydrochloride, and it does not combine with chloroplatinic acid.[16] Further reference to the literature showed that its *ortho* and *meta* isomers form salts more readily. An investigation of the reactions of these isomers with mercuric acetate showed not only that the reactions are completed within a very short time but at a comparatively low temperature. An attempt was then made to mercurize *p*-nitro-diethylaniline. This met with the same failure as that of the corresponding dimethyl derivative. That this failure to mercurize is not due simply to the fact that the *para* position is occupied was then demonstrated by the fact that *p*-nitro-monomethyl- and *p*-nitro-mono-ethylanilines are mercurized without difficulty, the mercury being oriented to the position *ortho* to the amino group.

These facts, taken in conjunction with the intermediates already isolated[17] and those obtained by Schoeller, Schrauth and Liese,[4] the complex salts of aromatic amines isolated by Klein[18] and the known general fact of the great affinity of mercury for an amino nitrogen, led us to adopt the hypothesis that mercurization proceeds first by the addition of the mercury salt to the amino group, *i. e.*, to form an ammonium salt. This ammonium salt then rearranges to a compound in which the mercuric radical is attached to carbon.[5]

From this theory of the mechanism of mercurization, one of the most logical deductions to be made is that, *of the aromatic amines only those which are capable of forming salts will mercurize*. Among other tests, one to which this deduction has been put was an attempt to mercurize trimethyl-phenyl-ammonium acetate, a compound in which the formation of a double salt with the mercuric acetate used is impossible. This com-

[15] Unpublished work of Kharasch, Lommen, and Jacobsohn.

[16] We found, however, that a hydrochloride of *p*-nitro-dimethylaniline could be prepared by passing dry hydrogen chloride into a benzene solution of the base.

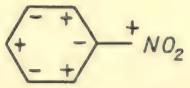
[17] Kharasch, Lommen, and Jacobsohn (unpublished work) have isolated the compound,  by treating an alcoholic solution of *m*-nitro-aniline with an aqueous solution of mercuric acetate.

[18] Klein, *Ber.*, **11**, 743(1878).

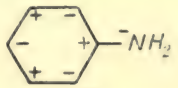
pound was not mercurized although a water suspension of dimethylaniline mercurizes within one or two minutes, with great evolution of heat.

The theory here advanced as to the mechanism of mercurization of aromatic amines adapts itself as already stated, quite readily to the explanation of the orientation of other groups upon substitution in the nucleus of aromatic amines.[19] Not only does it explain satisfactorily the cases of *ortho* and *para* substitution but, as will be shown later, it explains also the fact that a mixture of all three isomers is obtained when an aromatic amine is nitrated in conc. sulfuric acid solution, as well as the more recent observations of Vorländer[20] that, upon bromination and nitration, the quaternary ammonium salts yield only the *meta* derivative.

To explain these cases of *meta* substitution, Vorländer advances a theory of the benzene nucleus in which he assumes that the hydrogen atoms in the benzene molecule have positive tensions,[21] and that it is the tension within the nucleus which determines the position taken by the entering substituent. He assumes, further, that the nature of the tension on the group already substituted in the molecule exerts an influence on the arrangement of the tensions within the nucleus. Thus for nitrobenzene he assumes Structure I.

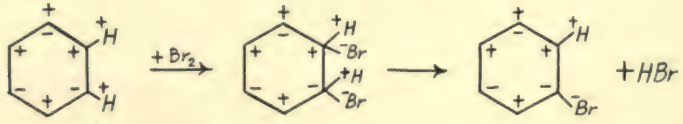


I



II

while, for aniline, Structure II is assumed. Further, substitution takes place by absorption with the subsequent loss of a molecule of acid or water[22] thus:



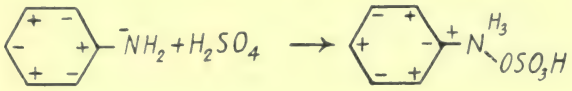
[19] It is self-evident that this theory is applicable also to such rearrangements as those of hydrazobenzene, diazo-aminobenzene, the Fischer and Hepp, etc. It is to be noted that, in all cases, where the rearranging compound is a weak base, the reaction must be carried out in the anhydrous media to insure salt formation.

[20] Vorländer, *Ber.*, **52B**, 263 (1919).

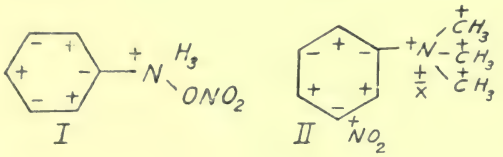
[21] In order to avoid committing himself to any theory as to the nature of valence, Vorländer uses the term "tension" and denotes the kind of tension by + and −. While he does not assume that they are electrical in nature, the properties assigned for them are such that, for the purpose of discussing substitution, there is little distinction to be made.

[22] It is rather peculiar that Vorländer does not take into account the fact that bromine is Br^+-Br^-, unless by an error in print, since the existence of positive bromine has been proved conclusively. (W. A. Noyes, THIS JOURNAL, **23**, 460 (1901); Stieglitz, *ibid.*, **23**, 796 (1901); Walden, *Z. physik. Chem.*, **43**, 385 (1903).

Vorländer makes the additional postulate, in order to explain the cases of *meta* substitution in aromatic amines, that, upon salt formation, there is a change in the tension throughout the molecule, *e. g.*, upon treating aniline with sulfuric acid, the following change is assumed to take place.



Such an assumption is entirely unwarranted. Not only are there no experimental facts upon which to base such an hypothesis, but the assumption made involves an upheaval taking place within the molecule, as well as a change of tension between the carbon and groups attached to it. Such a supposition as a change of tension would also demand that, in a compound such as aniline nitrate, analogous to aniline sulfate, the tension on the nitrogen bound to the phenyl group must be positive.



This compound should then, according to the views of Vorländer, give a *meta* derivative when dropped into conc. sulfuric acid. On the contrary, however, it gives only *o*- and *p*-nitro derivatives.[23] Similarly, the nitration of aromatic quaternary ammonium compounds demands, according to Vorländer, that the tension between the ammonium nitrogen and the carbon of the benzene nucleus be positive (II). This is hardly compatible with the fact that a water solution of the hydroxide of the compound (II) when evaporated gives a small quantity of trimethylamine and *m*-nitrophenol.[24] This theory must, therefore, be further modified if it is to be made to fit all the facts of substitution in aromatic amines.

Similarly, it has been necessary to make additional postulates to all previous theories in an attempt to build a workable theory of substitution. Yet, with all these postulates, none of the older theories can be made to fit all the facts of substitution. However, no new postulates need be made to the theory advanced by the present authors. Once it is assumed that all *ortho* and *para* substitution in the hydroquinoid nucleus of aromatic amines takes place through intermediate salt formation, it follows that, when such salt formation is impossible, as in the quaternary ammonium salts, substitution must take place in the *meta* position or, as in such special cases as that of mercurization, it is not accomplished at all.

[23] Bamberger, *Ber.*, **28**, 400 (1895).

[24] Staedel and Bauer, *ibid.*, **19**, 1939 (1885).

Not only does the theory advanced explain the case of *meta* substitution in the quaternary ammonium salts, but it explains also the fact that a mixture of all three isomers is obtained upon nitration of an aromatic amine in conc. sulfuric acid solution. It explains, further, the fact that the yield of the *meta* derivative increases with the amount of sulfuric acid used. Here it will be seen that an equilibrium mixture of both the amine and the ammonium salt exists in such a solution. Therefore, in such a mixture both types of substitution, that dependent upon intermediate compound formation, giving *ortho* and para derivatives, and the substitution of a salt yielding a *meta* derivative, are found. Again, as the volume of sulfuric acid is increased, the equilibrium will be shifted to form more of the sulfate, thus increasing the yield of the *meta* derivative.

Furthermore, the theory here advanced also offers an explanation of certain peculiar facts of rearrangement. For example, the rearrangement of β-phenyl-hydroxylamine[25] in alcoholic solution with hydrogen chloride as catalyst, gives not only *p*-amino phenetole but also *p*-chloroaniline, the more strongly electro positive chlorine atom rearranging. The theory advanced predicts that N-substituted aromatic amines, when treated with a catalyst capable of being oxidized by the group already substituted in the molecule will yield a derivative having the more strongly electro positive group in the *para* position.

It will be seen, therefore, that the present theory explains from a single standpoint the rearrangement and substitution in the case of aromatic amines, without the necessity of any additional postulates. It is also far more adequate for the explanation of these facts and furnishes a more workable hypothesis than any of the other theories of substitution.

Experimental Part.

Method used for Mercurization.

The method used for the preparation of the compounds herein described is, briefly, as follows. One mol of the compound to be mercurized was dissolved in a small volume of alcohol and boiled with an aqueous solution containing 0.9 mol of mercuric acetate, until a test portion gave no black precipitate of mercuric sulfide when treated with ammonium sulfide. This reaction mixture was filtered hot. Upon cooling, the acetate of the mercurized compound crystallized from the solution.

The filtrate from the above was treated with an aqueous solution of sodium chloride, whereupon the chloride of the mercury compound soon precipitated. The total yield of mercurized product calculated upon the basis of the mercuric acetate used, was almost quantitative.

An Attempt to Mercurize *p*-Nitro-dimethylaniline and *p*-Nitro-diethylaniline.—As stated in the theoretical part, several attempts were made to mercurize *p*-nitro-

[25] See, however, Stieglitz, *Am. Chem. J.*, **46**, 327 (1911

dimethylaniline, all of which met with failure. The method above outlined was used, as well as several other methods, none of which yielded a mercurized product. Similarly, attempts to mercurize *p*-nitro-diethylaniline were without success.

o-Nitro-p-Acetoxymercuri-dimethylaniline, ($C_6H_3(1)N(CH_3)_2(2)NO_2(4)HgO_2C_2H_3$.) —This compound was prepared from *o*-nitro-dimethylaniline and mercuricacetate. The reaction was completed after half an hour's boiling. The reaction mixture was filtered hot in order to remove the small amount of mercurous acetate formed in the reaction. It was then cooled and the precipitate collected on a filter. The precipitate was then washed with alcohol, and dried to constant weight *in vacuo* over sulfuric acid.

Analyses. Subs., 0.2759: dry N_2, 16.20 cc. (22° and 741 mm.). Subs., 0.2604: HgS, 0.1427. Calc. for $C_{10}H_{12}O_4N_2Hg$; N, 6.61; Hg, 47.23. Found: N, 6.63; Hg, 47.26.

The compound thus obtained is bright yellow; m. p. 160°. It is crystalline, and is soluble in the common organic solvents.

o-Nitro-p-Chloromercuri-dimethylaniline. ($C_6H_3(1)N(CH_3)(2)NO_2(4)HgCl.H_2O$).— This compound was washed well with water to remove sodium chloride and finally with alcohol, and dried *in vacuo* over sulfuric acid to constant weight.

Analyses. Subs., 0.2635: dry N_2, 15.60 cc. (21.5° and 741 mm.). Subs., 0.3233: AgCl, 0.1096. Calc. for $C_8H_{11}O_3N_2HgCl$: N, 6.69; Cl, 8.46. Found. N, 6.71; Cl, 8.38.

The substance is red and amorphous. It is soluble in acetone and in boiling alcohol. It melts at 185°, with decomposition.

***m*-Nitro - *p* - Acetoxymercuri - dimethylaniline,** ($C_6H_3(1)N(CH_3)_2(3)NO_2(4)HgO_2C_2H_3$).—This preparation was obtained upon treating *m*-nitro-dimethylaniline with mercuric acetate. A half hour's boiling was sufficient to carry the reaction to completion. For analysis, the compound was dried to constant weight over sulfuric acid.

Analyses. Subs., 0.2924: dry N_2, 17.05 cc. (20° and 734 mm.). Subs., 0.2940: HgS, 0.1610. Calc. for $C_{10}H_{12}O_4N_2Hg$:N, 6.61; Hg, 47.23. Found: N, 6.56; Hg, 47.23.

The compound is obtained in the form of brilliant orange needles. It is soluble in the common organic solvents; m. p. 140°.

***m*-Nitro - *p* - Chloromercuri-dimethylaniline,** ($C_6H_3(1)N(CH_3)_2(3)NO_2(4)HgCl$).— This compound was obtained from the filtrate of the preceding preparation.

Analysis. Subs., 0.3438: AgCl, 0.1237. Calc. for $C_8H_9O_2N_2HgCl$: Cl, 8.84. Found: 8.90.

The compound is red and amorphous. It is soluble in acetone and in boiling alcohol. It melts at 220°, with decomposition.

***p*-Nitro-*o*-Acetoxymercuri-monomethylaniline,** ($C_6H_3(1)NH.CH_3(4)NO_2(2)HgO_2C_2H_3$).—This compound was prepared from *p*-nitro-methylaniline and mercuric acetate. The product obtained was extracted with ether in order to remove unchanged *p*-nitro-methylaniline, and the residue was recrystallized from alcohol. A dark red solid remained which was insoluble in alcohol, but the quantity was too small to be analyzed.

Analyses. Subs., 0.2585: dry N_2, 15.6 cc. (21° and 736 mm.). Subs., 0.2981: HgS, 0.1673. Calc. for $C_9H_{10}O_4N_2Hg$: N, 6.84; Hg, 48.82. Found: N, 6.79; Hg, 48.38.

The compound forms small yellow crystals melting at 197° with decomposition. It is soluble in acetone and in hot alcohol to which a few drops of glacial acetic acid have been added. When treated with a conc. potassium hydroxide solution, a brick-red compound is formed. Upon dilution, the compound again becomes yellow.

p-Nitro-o-Chloromercuri-monomethylaniline, $(C_6H_3(1)N.H.CH_3(4)NO_2(2)HgCl\cdot H_2O)$.—This compound is obtained from the filtrate of the preceding compound.

Analysis. Subs., 0.2972: AgCl, 0.1036. Calc. for $C_7H_9O_3N_2HgCl$: Cl, 8.75. Found: 8.62.

The compound is yellow and crystalline. It is soluble in acetone and in boiling alcohol. It melts at 215°, with decomposition.

The position of the mercury in this compound was determined by the method of Dimroth.[26] The acetate was treated with two equivalents of a solution of potassium perbromide. The product was then extracted with ether and the extract purified from alcohol; m. p. 112–113°. When mixed with 4-nitro-2,6-dibromo-monomethylaniline, prepared by the method of Blanksma,[27] the melting point was not lowered. The use of only one molecule of the bromine in solution yielded no definite compound. Since the position could not be thus in dispute the dibromo derivative was therefore isolated.

p-Nitro-o-Acetoxymercuri-mono-ethylaniline, $(C_6H_3(1)N.H.C_2H_5(4)NO_2(2)Hg.O_2\cdot C_2H_3)$.—This compound was prepared from *p*-nitro-mono-ethylaniline, and was purified from the unchanged raw material by recrystallization from alcohol.

Analyses. Subs., 0.2641: dry N_2, 15.7 cc. (24.5° and 731 mm.). Subs., 0.3480; HgS, 0.1912. Calc., for $C_{10}H_{12}O_4N_2Hg$: N, 6.61; Hg, 47.23. Found: N, 6.56; Hg, 47.17.

It crystallizes from alcohol in small yellow crystals; m. p. 183°. It is also soluble in acetone. When treated with conc. potassium hydroxide solution, a brick-red compound is obtained. Dilution of the solution restored the original yellow color.

p-Nitro-o-Chloromercuri-mono-ethylaniline, $(C_6H_3(1)N.H.C_2H_5(4)NO_2(2)HgCl\cdot H_2O)$.—The compound was prepared in the usual manner. It is obtained in the form of an amorphous yellow solid; m.p. 218°, with decomposition. For analysis it was dried to constant weight over sulfuric acid.

Analysis. Subs., 0.4544: AgCl, 0.1573. Calc. for $C_8H_{11}O_3N_2HgCl$: Cl, 8.46; Found: 8.56.

It is soluble in acetone and in hot alcohol.

The position of the mercury in this compound was determined by the method used above for the corresponding methyl derivative. The melting point of the 4-nitro-2,6-dibromo-ethylaniline thus obtained was 68–71°. The melting point was not lowered when a mixture of the compound with known 4-nitro-2,6-dibromo-ethylaniline was used.

p-Nitro-o-Bromo-mono-ethylaniline, $(C_6H_3(1)N.H.C_2H_5Br(4)NO_2)$.—This compound was prepared by the addition of a solution of bromine in glacial acetic acid to a solution of an equivalent portion of *p*-nitro-mono-ethylaniline in the same solvent. After standing for about half an hour, the reaction mixture was diluted with water, and the precipitated compound collected on a filter. It was washed with water and a little alcohol. After crystallization from alcohol, the compound was dried to constant weight *in vacuo*. Yield, 90%.

Analysis. Subs., 0.2704: dry N_2, 26.50 cc. (17° and 750 mm.). Calc. for $C_8H_9O_2N_2Br$: N, 11.44. Found: 11.39.

The compound crystallized from alcohol in beautiful long yellow prisms which are highly refractive; m.p. 65° to 66°. It is insoluble in water but soluble in all common organic solvents.

When an alcoholic solution of the compound is treated with conc. potassium hydroxide solution, the alcoholic layer assumes a red color. Upon evaporation of the alcohol, or upon dilution with water, the yellow compound is again obtained.

[26] Dimroth, *Ber.*, **35,** 2033 (1902).

[27] Blanksma, *Rec. trav. chem.*, **31,** 271 (1902).

p-Nitro-*o*-Dibromo-mono-ethylaniline, $(C_6H_2(1)N.H.C_2H_5(2,6)Br_2(4)NO_2(1)$.—This compound was prepared in the same manner as the above monobromo derivative, in this case, with two mols of bromine. For analysis the compound was crystallized from alcohol. Yield, 90%.

Analysis. Subs., 0.2761: dry N_2, 21.2 cc. (21° and 744 mm.). Calc. for $C_8H_8O_2N_2Br_2$: N, 8.68. Found: N, 8.74.

This compound crystallized from alcohol in small yellow needles; m.p. 75–76°. It is insoluble in water, but soluble in all common organic solvents.

Upon treatment with a conc. potassium hydroxide solution, an alcoholic solution of the compound turns red. If the alcohol is evaporated, or the solution diluted with water, the color is restored to the original yellow.

An Attempt to Mercurize Trimethyl-phenyl-ammonium Acetate.—A solution of 0.5 mol of mercuric acetate was added to a solution of trimethyl-phenyl-ammonium acetate. Even after standing for two months at room temperature, no mercurized product could be isolated. The solution gave a heavy precipitate of mercuric sulfide when treated with hydrogen sulfide.

Upon boiling another portion of the solution for 24 hours, no change was observed. A heavy precipitate of mercuric sulfide was again obtained upon treatment with hydrogen sulfide.

Summary.

1. A theory of the mercurization of aromatic amines has been advanced, postulating that mercurization is preceded by addition of a mercuric salt to the amino nitrogen, with the subsequent rearrangement of the mercury to the *ortho* or *para* position. This theory, without any additional postulates, has been shown to apply equally well to the introduction of other groups in the benzene nucleus in the case of aromatic amines.

2. This theory has been shown to apply also to rearrangements such as that of Fischer and Hepp, the nitro-amines, sulfamic acids, etc.

3. The limitations to other theories of substitution have been pointed out.

4. The preparation of mercury derivatives of *p*-nitro-methylaniline, *p*-nitro-ethylaniline, *o*-nitro-dimethylaniline, and *m*-nitro-dimethylaniline, and the preparation of *o*-bromo and *o,o*-dibromo-*p*-nitro-ethylanilines have been described.

CHICAGO, ILL.

AN INVESTIGATION ON THE ACID-FASTNESS OF TUBERCLE BACILLI II*

By Binzi Suyenaga, M. D.

Chicago

In the previous report on this same subject, I showed that rapid transfer of the border or younger portion of colonies of Kl, a saprophytic non-virulent strain of tubercle bacilli, did not materially affect its acid-fastness. In other words, I was unable to prove by this method that younger cultures are much less acid-proof than older ones. I also changed the metabolic conditions of the culture by using different reactions, which had no marked effect on the acid-fastness, and by non-nutrient media and the amœbia media used by Wherry both of which greatly reduce the acid-fastness without absolutely destroying it. The question that especially needs answer is whether, in any such culture as Kl, in which we find many acid-fast, but also many non-acid-fast bacilli, we have a culture consisting of two distinctly different strains or whether the bacilli merely differ from each other as a result of age, metabolic conditions or degenerative changes. If we have two distinct strains, one may be a mutation strain or the two may be foreign to each other and live together symbiotically.

Two possible methods suggested themselves which might separate the non-acid-fast from the acid-fast strain, if the two existed as separate and distinct strains in the saprophytic culture used by me. Churchman had suggested that gentian violet and methylene blue, though in different degrees, might separate from each other Gram positive and Gram negative strains which were growing together. He showed that the Gram positive strains were completely inhibited by the dyes. It was my hope that the same dyes might separate the acid-fast from the non-acid-fast strain by inhibiting or killing one of them. The bactericidal power of these dyes was tested on Kl and other acid-fast organisms, such as avian and frog tubercle bacilli and leprosy bacillus and on a number of non-acid-fast rapidly growing organisms (B. prodigiosus, B. subtilis, B. coli and staphylococcus aureus). The amount of the dye necessary to make the desired dilution was added to a broth culture which was then incubated for two hours and kept at room temperature from three to four hours. A definite amount was then transferred to plain agar slants. Neither gentian violet nor methylene blue had any bactericidal action except on staphylococcus aureus. This was killed by 1 to 10,000 gentian violet and mostly by 1 to 5000 of methylene blue. In another experiment B. subtilis was killed by gentian violet in a dilution of 1 to 1000 and B.

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coli and B. prodigiosus by a dilution of 1 to 200. In the case of the acid-fast organisms which, after exposure to the dyes were inoculated on glycerol agar, Kl seemed not to be killed by any dilution used, but the avian and frog tubercle bacilli were killed by all dilutions used of both dyes, the highest being 1 to 1000, while the leprosy bacillus was killed by 1 to 500 and much delayed in its growth by 1 to 1000. A concentration of 1 to 100,000 of either gentian violet or methylene blue using the Churchman divided plate method seemed to have no inhibitory effect on the growth of any of the organisms on plain agar except B. subtilis and staphylococcus aureus, while 1 to 1000 gentian violet completely inhibited the growth of Kl, timothy B., grass B., mist B., smegma B., and B. subtilis and staphylococcus aureus. Methylene blue 1 to 1000 completely inhibited the growth of timothy B. and B. subtilis and delayed the growth of Kl. On glycerol agar, the results are somewhat different. Kl shows no inhibition even at 1 to 1000 of either dye, while avian and frog bacilli are inhibited completely by 1 to 100,000 of gentian violet and by 1 to 1000 methylene blue. Leprosy bacillus is completely inhibited by 1 to 10,000 gentian violet and by 1 to 1000 methylene blue.

It was found on the divided plates that frog bacilli and avian bacilli which are least acid-fast were most susceptible to the action of the dyes, that they grew only on the undyed portion and their growth never extended near the line separating the dyed and undyed portions, while Kl, leprosy and smegma bacilli grew entirely across the plate, if the concentration of the dye did not exceed 1 to 100,000. In order to determine whether the bactericidal and inhibitory action of these dyes would prove a feasible method for separating acid-fast from non-acid-fast organisms, cultures of Kl after exposure to gentian violet and methylene blue were stained by the Ziehl-Nielsen method and it was found that acid-fast and non-acid-fast organisms were still mixed.

The second method which suggested itself for separating these two types of bacteria, which should succeed if they represent two separate and distinct strains, is the method used by Petroff for isolating tubercle bacilli from other organisms which may occur in the sputum. Kl culture was exposed for from ten minutes to five hours to sodium hydroxide, then neutralized and inoculated on Petroff's egg media and on glycerol agar. Sodium hydroxide, 1.5 per cent. killed all organisms even in ten minutes, so that no growth occurred. Sodium hydroxide, 0.5 per cent. did not kill all in one hour but did in two hours. A virulent culture was also exposed to 0.5 per cent. sodium hydroxide. No growth occurred on the glycerol agar slants even after ten minutes' exposure, but there was growth on all the Petroff media containing 1 to 10,000 gentian violet, even after five hours. The mixture of acid-fast and non-acid-fast bacilli in the tubes, in which growth occurred after the exposure to sodium hydroxide, shows that these two types of bacilli could not be separated by this method and suggests therefore that they are not distinct strains.

If this is true, they must probably be phases in the growth of the tubercle bacillus. Ehrlich, Marmorek, Klein and others regarded the non-acid-

fast forms as merely the youngest phase in the development of the tubercle bacillus. Many have thought that the fatty sheath which is supposed to contribute to the acid-fastness of the bacillus develops later. By increasing the rapidity of the growth and thus developing a strain which should consist only of young bacilli I endeavored without success in my earlier work to show that the age of the culture had much to do with its staining qualities. Metabolic conditions seem, from my former report, to affect markedly the acid-fastness of these organisms, since media of low nutritive value greatly diminished their acid-fastness. Degenerative conditions due to the age of the culture and the consequently lowered nutritive value of the media may be a possible factor in increasing the number of non-acid-fast bacilli. But that question has not as yet been investigated.

CONCLUSIONS

1. Since it has not proved possible by the methods used to separate an acid-fast strain from a non-acid-fast, it is probable that we have here no distinct strains, either mutation or symbiotic.

2. Since the marginal growth of Kl seems to contain more non-acid-fast organisms than the central portion and since some non-acid-fast organisms occur even in virulent cultures, it seems probable that the younger bacilli may for a short time be non-acid-fast. The question whether the non-acid forms may be degeneration forms has not been investigated.

3. Gentian violet has a stronger bactericidal and inhibitory power than methylene blue over saprophytic acid-fast and some Gram positive and Gram negative organisms.

4. There is considerable difference between the bactericidal and the inhibitory power of these antiseptic dyes.

5. Of the acid-fast bacilli, the less acid-fast seem more susceptible to the action of gentian violet and methylene blue.

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A METHOD OF FOLLOWING REACTION CHANGES IN CULTURES OF ACID-FAST BACTERIA

ESMOND R. LONG AND AGATHA L. MAJOR

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In that graphic expression of acid and alkaline changes taking place in the culture media of tubercle bacilli which has for years been designated as the Theobald Smith curve (1), we are furnished with highly suggestive leads on the metabolism of this organism. From a protein-glycerol mixture both alkali and acid are produced at rates more or less constant for a given strain. The chemistry of the change has not yet been definitely worked out.

As orginally developed, the method of preparing the curve required large numbers of duplicate culture flasks, two or more of which would be taken for titration each week and then discarded. About four months were required for the complete cycle. The method was thus long and tedious, and required a large amount of incubator space for a single determination. This was before the days of ready determination of reaction by estimation of hydrogen ion concentration

A few years ago Frothingham (2) followed the reaction change in a culture of a human type bacillus, H37 of the Saranac Laboratory, using the potentiometer, and obtained a curve comparable to the one usually obtained by the old titration method, with a range of reaction which may be calculated from his figures as pH 8.15 to 5.52. A little later, the same author (3) studied the growth of tubercle bacilli on media to which small amounts of indicator had been added. Using brom-cresol-purple, he found that glycerol broth culture media of human type bacilli tended to become acid, while those of the bovine type remained alkaline. He concluded that brom-cresol-purple and litmus may be useful in determining H ion concentrations of culture media of tubercle bacilli and in distinguishing human and bovine types.

Recently Dernby (4) determined the limits of growth of a strain of tubercle bacillus (horse) as pH 6.0 and 7.6, and the optimum concentration for growth, pH 6.8 to 7.2. One of us (5) noted initial growth

between pH 6.4 and 7.8 for a human type bacillus with no apparent inhibition at 5.4 or 7.8. It is thus able to carry on its biological activities through a fairly wide range of reaction.

We submit, herewith, a method similar to the one used by Frothingham, in which the change in reaction can be followed with a high degree of accuracy between the limits pH 6.4 and 8.4, using phenol-sulphone-phthalein, which passes through a characteristic series of color change within those limits, as indicator. Four test tubes 1 inch wide, each containing 10 cc. of glycerol-peptone broth or any other liquid medium, neutral in reaction (pH 7.0), plus 0.001 per cent phenol-sulphone-phthalein, are inoculated with plaques of tubercle bacilli, approximately 0.75 cm. in diameter, sealed with tin foil, and incubated at 37.5°C. Once a week the tubes are taken out and the color compared with standards of known reaction containing the same concentration of indicator. We use the Dernby and Avery (6) comparator, placing a tube of broth back of the standard when we are determining broth reactions, to compensate for the color of the medium. Apparently, at this concentration the indicator does not inhibit the growth of these organisms. Care must of course be taken that the plaque remains floating. The reactions of the four tubes are averaged in plotting the curve.

In developing the method, we have carried out a large number of determinations on rapidly growing saprophytic acid-fast organisms as well as tubercle bacilli, and have grown these organisms on culture media containing a single amino acid or acid amide as the sole source of both carbon and nitrogen, from which they must build the entire amount of their proteins, lipoids and carbohydrates. We have thus obtained considerable light on the metabolism of this group. A series of our reaction curves is given in twelve appended charts. The saprophytes were cultivated for about one week and tubercle bacilli for two months. By the method it is aimed to save time, trouble and the necessity of running large numbers of bulky cultures for a single curve.

Three strains of tubercle bacilli and two strains of grass bacilli and one of smegma bacillus were studied. One of the grass bacillus strains is called "timothy bacillus" because it has for years gone under that name in the Saranac Laboratory stock, to which it was originally supplied by Moeller. It differs from the other one in its reaction curve, as is seen from the figures, and also in that it is decidedly chromogenic. H37, and B1 are old, but virulent, cultures of human and bovine type tubercle

bacilli. H104 is a highly virulent human type tubercle bacillus isolated from sputum in June, 1920. As will be noted, the initial reaction varies a little; in all cases the intention was to start the experiment at pH 7.0, but occasionally autoclaving changed the reaction slightly, and the experiment was proceeded with at the new reaction.

SUMMARY OF RESULTS

Saprophytes: On glycerol-peptone broth the grass bacillus caused a slight acidity, the timothy bacillus a distinct alkalinity, and the smegma bacillus little change, in spite of good growth. On alanine the grass bacillus caused a distinct acidity, the timothy bacillus a distinct alkalinity, and the smegma bacillus little change. It will be noted that the results for glycerol-peptone broth and alanine are about the same. This is to be expected, in view of the fact that peptone consists almost entirely of polymerized amino acids like alanine: the same reaction change would result in both from a given cause. The glycerol, highly necessary as it is for tubercle bacilli, probably plays little part here, as these organisms thrive on peptone alone. On propionamide, which differs from alanine in the location of its amino group, all organisms caused a marked alkalinity.

Tubercle bacilli: H37 on glycerol broth gave the curve generally considered to be characteristic of human type bacillus, a primary alkalinity followed by a progressive acidity. It is to be noted that the change in reaction occurred much sooner than is the case in the flask titration method. This is presumably due to the small volume used, 10 cc., which would respond quickly to the elaboration of small amounts of acid and alkali. We feel that this rapidity is a distinct advantage of the method. B1 also gave the curve said to be characteristic for the bovine type bacillus, an initially increasing alkalinity, which later decreased, but not to the point of neutrality. H104 culture became alkaline and remained so for two months. No change was to be noted in any of these cultures after that time. The curve of H104 was thus not of the classic human type. It is simply one of the numerous exceptions.

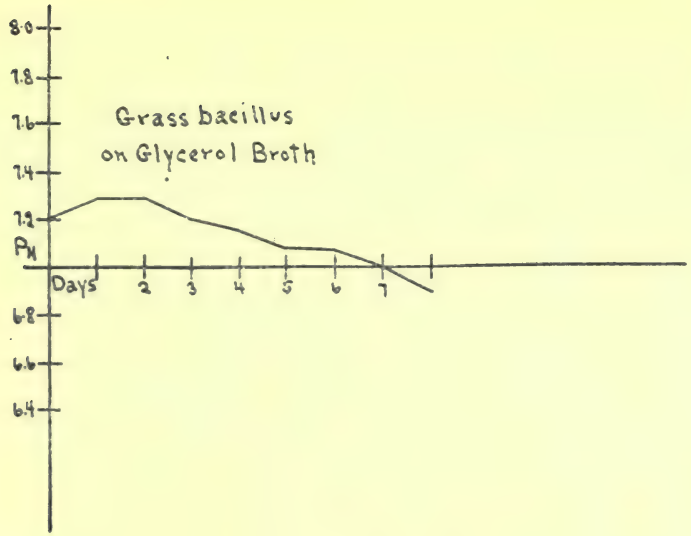
The chemistry of these changes will not be gone into at the present time, as this paper is of the nature of a preliminary report on method. We are concerned now with simplifying old methods and establishing that characteristic curves can be secured on simple media of known composition with some organisms. So far, characteristic results for tubercle

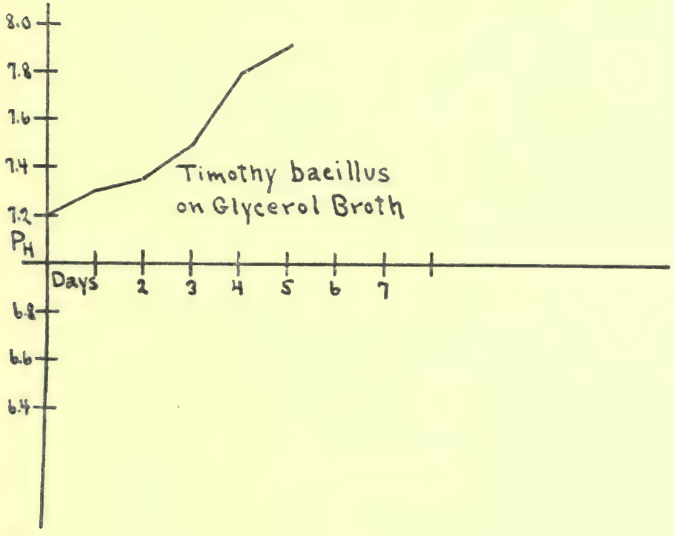
bacilli on simple media have not been secured. Our problem is to modify such media enough to promote good growth and yet not enough to complicate chemical interpretation. It is the chemistry of the change which we wish to understand, for its bearing on the organism's metabolism.

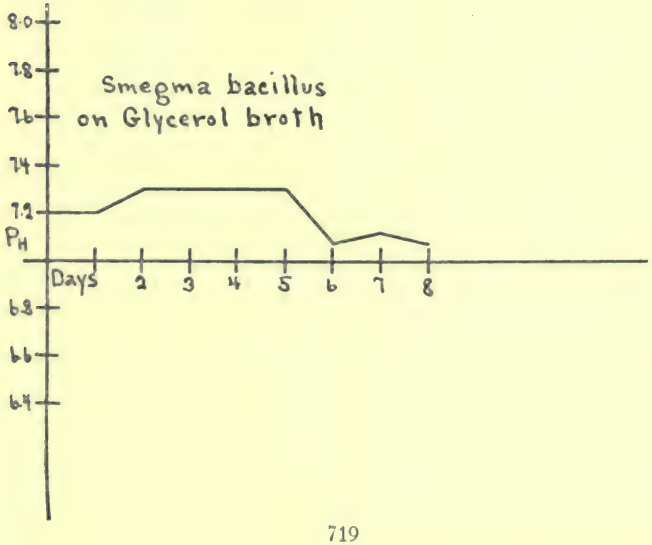
This investigation was aided by a grant from the National Tuberculosis Association.

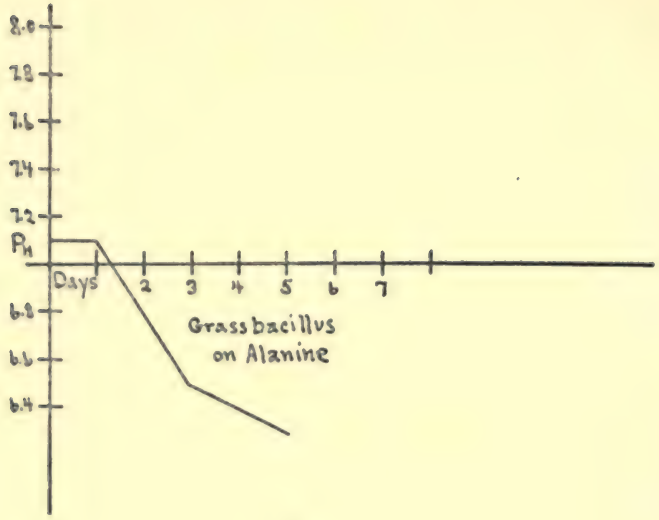
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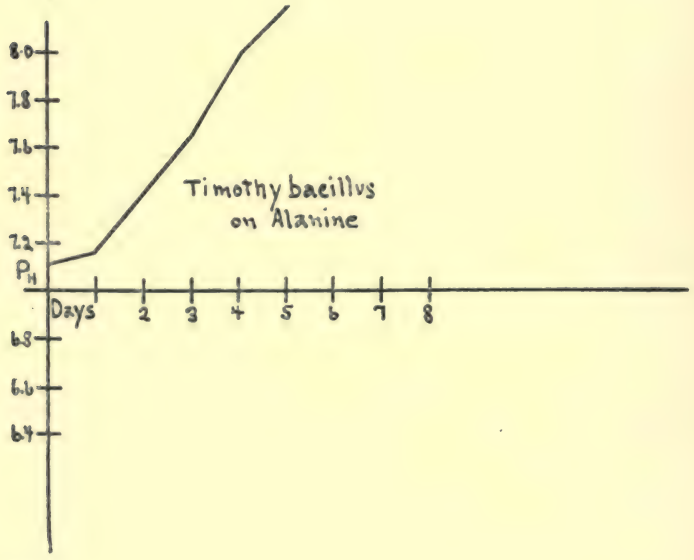
(1) SMITH: J. Med. Res., 1910, xxiii, 185.
(2) FROTHINGHAM: Ibid. 1917, xxxvii, 269, and 1918, xxxix, 153.
(3) LONG: Amer. Rev. Tuberc., 1919, iii, 86.
(4) DERNBY: Ann. de l'Inst. Pasteur, 1921, xxxv, 277.
(5) DERNBY AND AVERY: J. Exp. Med., 1918, xxviii, 345.

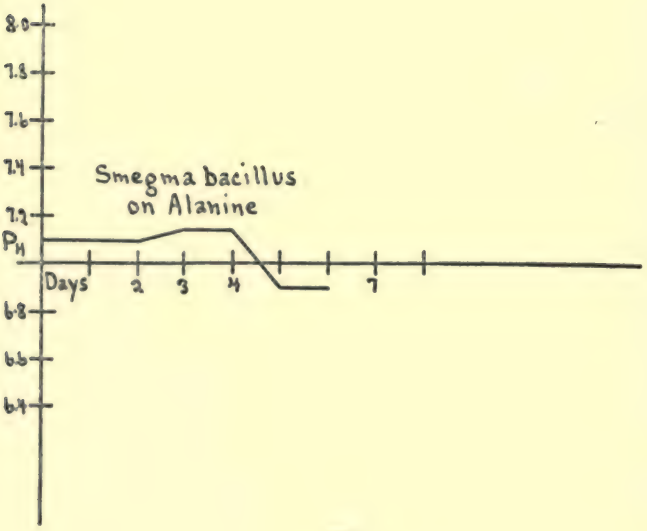

8.0
7.8
7.6
7.4
7.2
PH
Grass bacillus
on Glycerol Broth
Days
2
3
4
5
6
7
6.8
6.6
6.4

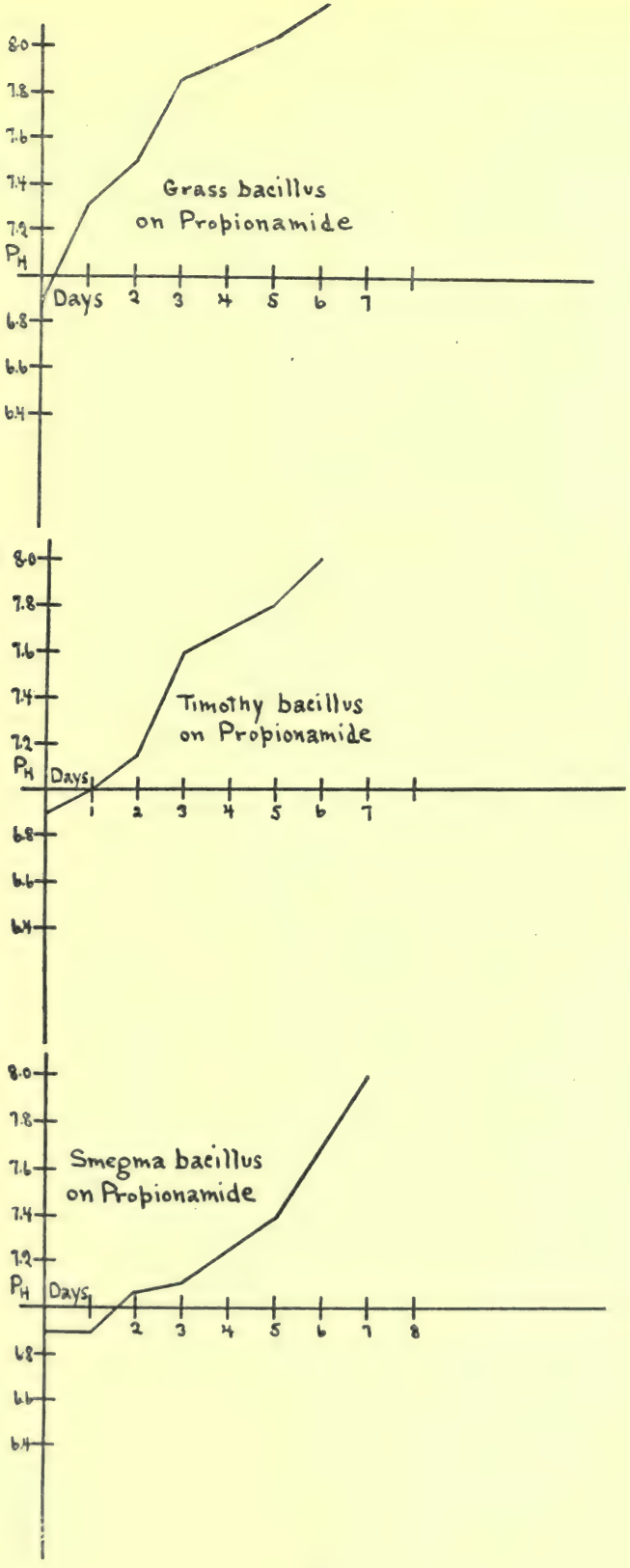

8.0
7.8
7.6
7.4
7.2
PH
Timothy bacillus
on Glycerol Broth
Days
2
3
4
5
6
7
6.8
6.6
6.4

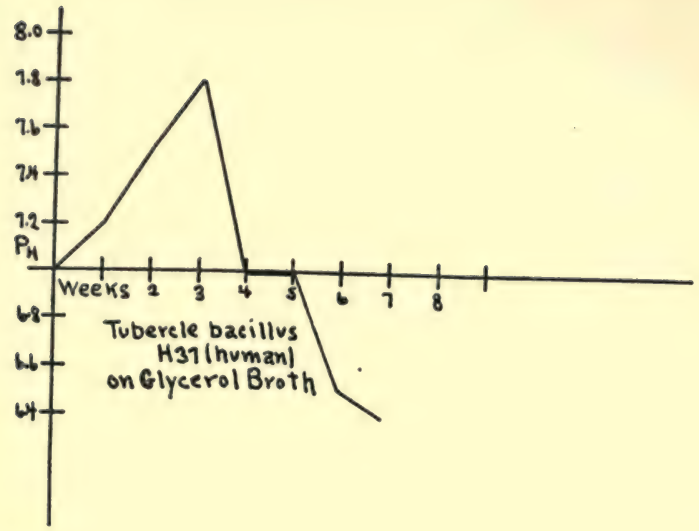

8.0
7.8
7.6
7.4
7.2
PH
Smegma bacillus
on Glycerol broth
Days
2
3
4
5
6
7
8
6.8
6.6
6.4

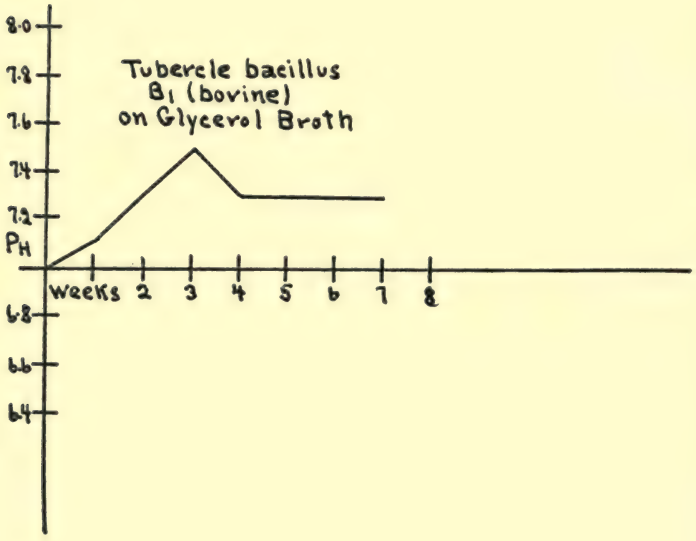

8.0
7.8
7.6
7.4
7.2
PH
Days
2
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6.8
6.6
6.4
Grass bacillus
on Alanine

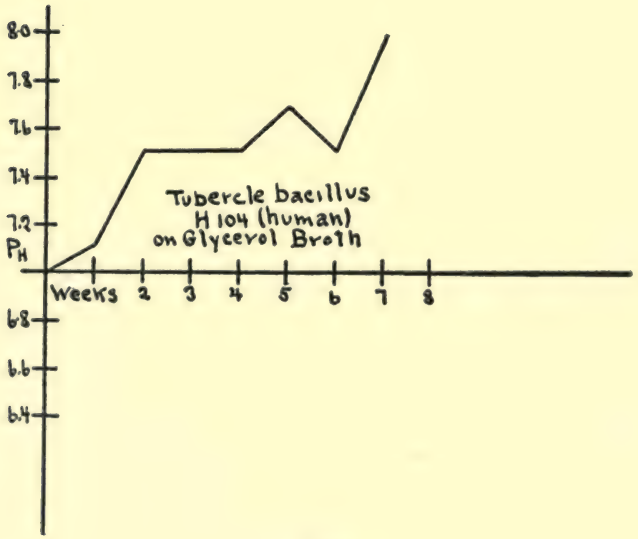

8.0
7.8
7.6
7.4
7.2
PH
Timothy bacillus
on Alanine
Days
2
3
4
5
6
7
8
6.8
6.6
6.4


8.0
7.8
7.6
7.4
7.2
PH
Smegma bacillus
on Alanine
Days
2
3
4
7
6.8
6.6
6.4


8.0
7.8
7.6
7.4
7.2
PH
Grass bacillus
on Propionamide
Days 2 3 4 5 6 7
6.8
6.6
6.4
8.0
7.8
7.6
7.4
7.2
PH
Timothy bacillus
on Propionamide
Days
1 2 3 4 5 6 7
6.8
6.6
6.4
8.0
7.8
7.6
7.4
7.2
PH
Smegma bacillus
on Propionamide
Days
2 3 4 5 6 7 8
6.8
6.6
6.4


8.0
7.8
7.6
7.4
7.2
PH
Weeks 2 3 4 5 6 7 8
6.8
6.6
6.4
Tubercle bacillus
H37 (human)
on Glycerol Broth


8.0
7.8
7.6
7.4
7.2
PH
Tubercle bacillus
B1 (bovine)
on Glycerol Broth
weeks 2 3 4 5 6 7 8
6.8
6.6
6.4


8.0
7.8
7.6
7.4
7.2
PH
Tubercle bacillus
H 104 (human)
on Glycerol Broth
Weeks 2 3 4 5 6 7 8
6.8
6.6
6.4

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CHEMICAL PROBLEMS IN THE BACTERIOLOGY OF THE TUBERCLE BACILLUS[1]

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An able investigator, Dr. Kendall, once defined bacteria, or partially defined them, as chemical reagents. The characterization is by no means far fetched, as we see when we stop to consider the multitude of reactions carried out sharply and completely by different bacteria, reactions duplicated exactly by ordinary analytical methods with common chemical reagents in the laboratory Consider the phenomenon of deaminization. If I add to the substance alanine, which has an amino or NH_2 group in its structure, a small amount of nitrous acid and warm the mixture, nitrogen is split off and a hydroxyl group substituted. The process is deaminization. The same end result may be secured through the action of a great variety of bacteria. Just how they accomplish the result we do not know. We speak somewhat vaguely of enzyme action, with notions far from clear on intermediate steps; yet the end result we know with certainty.

Take for another example the production of histamine from histidine. The latter is an amino acid and like all amino acids contains a carboxyl or COOH group. Under the action of some bacteria, notably certain strains of B. coli, CO_2 is split off and imid-azol-ethylamine produced, histamine, one of the most potent poisons known. Some of the same product may be produced from histidine in the laboratory by the simple process of dry distillation, CO_2 being split off at the high temperature.

Or consider the action of common yeast on dextrose. Through the enzyme action of the yeast we have produced—if we agree with Neuberg —pyruvic acid and atomic hydrogen. From the former, carbon dioxide is evolved with the production of acetaldehyde, which in turn is reduced by the aforesaid hydrogen with the production of ethyl alcohol,—no mean accomplishment for any chemist these days.

[1] Read at a joint session of the Clinical and Pathological Sections, at the Seventeenth Annual Meeting of the National Tuberculosis Association, New York City, June 16, 1921.

Now much as I respect the analytical powers of bacteria I am more profoundly impressed by their aptitude for the reverse process, synthesis, their power of building up complex structures from the simplest of materials. I think of them as chemical machines especially adapted for synthesis on a grand scale. Of course, this ability is common to all forms of life from a baby down, but the process is followed more readily in the simple unicellular forms. The characterization as chemical machinery for synthesis holds for parasites and saprophytes, the difference in the two being entirely in their reaction to environment. If I may attempt a definition, too, I will say that a parasite is a mixture of proteins, capable of synthesizing more protein within the tissues of a living being; protein being the substance, according to the best information available at the present time, characteristic of life; and the rapid construction of that protein by small masses of the original protein constitutes virulence, chemically considered.

Unfortunately we are unable to catch any of the midstages in this remarkable process, although we can detect a good many in the reverse process, that of tearing down a protein molecule. We are able to set up a chemically well defined meal before a given bacterium, and determine with a high degree of accuracy what it takes and what it leaves, and then we are able to analyze the product and say—with far less accuracy—what it has done with what we provided. But how it put the structure together from the simple substances we gave it remains a puzzle.

I have been interested for some time in the utilization of certain food stuffs by the tubercle bacillus. Here is an organism that takes *us* for its food material and builds up its peculiar proteins out of the disintegration products of our proteins. I shall never forget how deeply impressed I was, when I was assisting in the work at the Reception Hospital at Saranac Lake, at the phenomenon of a twenty year old girl bringing up a quart of sputum a day, every one-twelfth oil immersion field of which contained hundreds and hundreds of living tubercle bacilli. There was synthesis, and parasitism, and virulence.

Now what is this tubercle bacillus, whose capacity for synthesis is so destructive to the human host? Chemically considered it is a mixture of proteins, nucleoproteins, and perhaps lipoproteins and glycoproteins, in a cell which contains a high amount, as compared with other bacteria, of certain peculiar lipoids. Because of this characteristic high fat content and its relation to the phenomenon of acid-fastness, an enormous amount of work has been done on this phase of the chemistry of the

organism, and, I think, in proportion to the time, effort, and money spent on it, with the least success. Not that much good work has not been done, or that good men have not attacked the problem. I simply mean that lipoids are physically of such consistency that most of them have never been separated sharply, and purified and identified. But I have high hopes in this direction. Dr. Levene, who has done such splendid work in this field recently, tells me that we may look forward to the elaboration, within a very few more years, of methods which will make the analysis of lipoid mixtures a fairly exact procedure. In the meantime we have of course acquired much valuable information, even if it is far from complete. We know that the waxy mixture contains a good deal of true fat, and a high proportion of an alcohol of high molecular weight, called "mykol" by Sakae Tamura, and that phosphatids are present in considerable amount. I want to know more about these last substances, the phosphatids. I believe that the phosphorus problem has been highly neglected in bacterial chemistry in general. Here is a field which must be carefully cultivated when the new methods arrive.

I should like to take a vote in this audience to see how many consider the tubercle bacillus of plant, how many of animal nature. I have had it in mind for some time to apply certain chemical tests to this organism, to gain information on this perhaps academic point. As you all know the chemical distinction between plant and animal life is far from absolute; yet there are some lines on which broad generalizations on differences have been laid down. One of these respects the insoluble carbohydrates. In plants, the woody forms of life, we have such substances as cellulose, hemicelluloses and starch. In the lower forms of animal life, insects, crustaceans, etc., we have, in the hard parts, carbohydrates made up just as in plant life from polymerized small molecules, but with this difference,—that they contain nitrogen. In them, not glucose molecules as in starch, but glucosamine molecules, are piled up to make what is known as chitin. Now chitin has been described in several bacteria. Iwanoff was among the first to detect it, noting its presence in several forms, including the anthrax bacillus. As good a man as Ruppel, whose early article on the proteins of the tubercle bacillus is classic, suggests that chitin or a similar substance might be responsible for the acid-fastness of the organism. Helbing believed the same thing. The evidence was poor. But there is a problem to be worked out at any rate. Of course everything under the sun has been referred to as responsible for the phenomenon of acid-fastness, in

almost total neglect, it seems to me, of the well known fact that an extremely finely powdered mass of tubercle bacilli ground, let us say, in a ball mill, is non-acid-fast. Presumably such a mass contains the same chemical constituents as the unground bacilli. The phenomenon of colloidal dispersion of the different constituents has not been thoroughly taken into account. The problem should be attacked by the physical as well as the organic chemists.

The protein of the tubercle bacillus has been considered essentially nucleoprotein. I think more or less inaccuracy has been involved in the decision, although there is no question that a good deal is nucleoprotein. But the test for nucleoprotein has too often been the presence of phosphorus in a crude protein mixture having the solubility characteristics of known nucleoproteins. In this connection I may say that just the other day in my laboratory we found a substance in defatted tubercle bacilli, which was rich in phosphorus, was soluble in water and alcohol, insoluble in toluene and acetone, and which was organic and gave a mercury salt, but gave none of the protein reactions. I was looking at the time for alcohol soluble proteins, which at the present time, because of the work of Osborne, are attracting considerable attention.

Some months ago, instead of using phosphorus as the indicator for the amount of nucleic acid present, I used substances far more definitely characteristic of nucleic acid, namely, the purine bases, and from the amounts found calculated that the nucleic acid content of the bacillus was between 2 and 3.5 per cent of the dry weight. This was smaller than I expected, and led me to believe that a higher proportion than I had originally supposed was simple protein. Through the coöperation of that excellent chemist, Dr. Treat B. Johnson, I expect to have another check run on this, using not the purine bases this time but other substances also highly characteristic of nucleic acid, the pyrimidine bases. Further data on a point I mentioned a few moments ago may be furnished by this investigation. Curiously enough, as far as we know now, there are two distinct nucleic acids and just two, one of these being found in plant forms, like yeast and wheat, the other occurring in animal tissues, like pancreas or thymus gland, the chromosomes of animal nuclei in general presumably. They are a good deal alike. The structure is the same. Each is built upon phosphoric acid linked with two pyrimidines and two purines by carbohydrate bridges. The purines are the same, guanine and adenine, but the pyrimidines vary and the

bridge is different. Both forms are said to contain the pyrimidine cytosine, but in the plant acid the other pyrimidine is apparently uracil, while in the animal form it is thymine. And the carbohydrate bridge is in the plant acid a pentose, and in the animal a hexose. Now I am going to have the nucleic acid of the bacillus more thoroughly investigated. Through the courtesy of Parke, Davis & Co. and Mulford & Co. and Dr. Dorset of Washington, I have collected about three kilograms of dry tubercle bacilli, which are to be analyzed for certain things. Dr. Levene has promised to get out the nucleic acid and determine if its sugar is a pentose or a hexose, in series with his other valuable reports on nucleic acids. Dr. Johnson will supervise the work of a reliable pyrimidine chemist, who also will get out the nucleic acid and determine what pyrimidine bases are present in it. I may say that the presence of thymine has been recorded by several investigators, and cytosine and uracil have been looked for, but the results are incomplete and confusing.

There is desire for much investigation on the proteins present, because, of course, the phenomena of hypersensitiveness are the result of protein sensitization. It seems ridiculous that having known that fact for years we have so far failed to devote much attention to learning of what type those proteins are. We know the qualitative composition, to a certain extent, of the hydrolyzed proteins—Tamura has furnished us with a list of some of the amino acids present—but we do not know what the particular types of proteins present are. I plan to use some of my six pounds of tubercle bacilli on this problem.

Leaving the subject of the chemical composition of the tubercle bacillus for one with which I have more personal acquaintance, I would like to say something about the metabolism of the tubercle bacillus and some other acid-fast organisms. One of the best leads I know about in this field was furnished fifteen years or more ago by Theobald Smith, who noted that in the cultivation of tubercle bacilli on glycerol broth the media went through first an alkaline, then an acid phase. These changes have not been accurately accounted for chemically; partly, I believe, because we have used too complex media for the purpose. Commercial peptone is a hopeless mixture of things, and meat extract is much worse. I am engaged at the present time in running Theobald Smith curves on media of simple, known chemical composition, and trying now to understand the first or alkaline phase. I can think of just three ways in which culture media, glycerol-peptone broth, let us say, can become alkaline as the result of bacterial growth. One is by the

splitting of amino acids or acid amides, which of course make up the bulk of peptone, into ammonia and an organic acid, with destruction of the acid or its incorporation into bacillary substance, and excretion, or at least slower utilization, of ammonia, leaving a preponderance of this substance in the medium.

The second is by the decarboxylation of these amino acids, carbon dioxide being driven off and alkaline substances, the amines, being left. This theory was quite attractive to me two years ago, because at that time I believed the tubercle bacillus would grow on methyl and ethyl amines as sources of nitrogen, and thought that it might be by this amine route that the corresponding amino acids, namely glycocoll and alanine, were broken down. I have since concluded that those results were due to impurities, chiefly ammonium chloride, in the preparations, and that the tubercle bacillus will not grow on amines, and that therefore these are probably not evolved in its katabolism of the amino acids of its food supply. The point is one of considerable importance, as the amines are very potent substances, physiologically considered. Take some examples. In the suprarenal gland we apparently have the amino acids, tyrosine and phenylalanine, decarboxylated and otherwise slightly modified and converted into the highly active epinephrin. At least one investigator believed that the hypophysis decarboxylates some of the histidine which reaches it and turns back into the blood stream the very important substance histamine. If the tubercle bacillus is one of the decarboxylating organisms, might we not think of the tubercle as a ductless gland, also turning into the blood stream some of these decarboxylated amino acids? The idea has received some attention, especially with reference to the production of histamine. I have proved that the tubercle bacillus is able to use histidine as its sole source of nitrogen, and Drs. Koessler and Hanke by their new methods have shown that histamine is not produced from histidine present in the medium, but that one of the corresponding deaminized organic acids, imidazolpropionic, pyruvic or lactic acid, is. These are innocuous substances.

The third possibility I had in mind to account for the alkaline tide is the withdrawal of phosphoric acid from the neutral phosphates present, for the construction of nucleoproteins and phospholipins, leaving the bases behind. I am convinced that this process plays some rôle, although it is probably small in comparison with possibility number one, which I outlined a moment ago, ammonia production.

Before leaving the subject of the Theobald Smith curve I should like to describe a simple method of following it, which saves time, and an immense amount of incubator space, obviating the necessity for the large number of cultures used where titrations are carried out each week. The method is not new, a similar one having been first used by Frothingham. I carry out the procedure as follows: A plaque of tubercle bacilli, approximately 1 cm. in diameter, less if necessary, is planted on 10 cc. of broth in a test tube, 1 inch in diameter, the broth being exactly neutral in reaction at the start, being either glycerol-peptone broth or a special medium, and containing 0.001 per cent of phenol-sulphonephthalein, which passes through a characteristic series of color changes between the hydrogen ion concentrations pH 6.4 and 8.4. At weekly intervals the tubes—I run the experiment in quadruplicate—are compared with standards containing the same concentration of indicator at different reactions, and the reaction curve is plotted. When 10 cc. amounts are used in well sealed tubes the whole procedure takes about two months. Obviously, it is much simpler than the titration method. This work is not quite ready for publication, but I may say that for H37 and B1 on glycerol broth, respectively human and bovine type strains from the Saranac Laboratory, I have secured curves quite comparable to those usually obtained by titration methods. The results on media of known composition are not yet as characteristic as I had hoped; and yet I believe that it is only by the use of such media that we shall ever be able to interpret the phenomenon. With certain saprophytes, such as smegma and grass bacilli, where glycerol need not be added, where a single amino acid, such as alanine, or acid amide, as propionamide, suffices for both carbon and nitrogen, striking results have already been obtained, which will be published in the near future.[2] They furnish very clear indications as to the protein metabolism of these organisms.

Inasmuch as the amino acid alanine can be considered as a type form for many members of the group, the ring compounds tyrosine, phenyl alanine, tryptophane and histidine, being simply substitution products of it, I am concentrating my attention on the products obtained from it, being at the present time engaged in the analysis of 2 litres of an alanine mixture upon which H37 was grown for one month. I am hoping to detect either ammonium lactate or ammonium pyruvate,

[2] See this number, p. 715.

ammonium propionate and ethyl amine, the other two possible decomposition forms, having been apparently ruled out.

In the investigation on metabolism up to the present time, I have run perhaps five thousand cultures of acid-fast organisms on media of known composition. Little of this has been published, as I confess that so far I have only accumulated many details without being able to establish broad general principles with which the details may be correlated. Bacteria, while chemical reagents, are very complicated reagents, not always of standard purity, so that many times the expected reaction does not take place and is replaced by another. Even so, I believe them more reliable reagents in our work than the higher forms of life, such as guinea pigs.

A brief summary of the details which have been observed should include the following facts. In general I have found, using human, bovine, and avian tubercle bacilli, frog, turtle and fish bacilli, leprosy bacilli, smega bacilli, and several strains of grass and mist bacilli, that nitrogen is readily abstracted from the amino acids alanine, leucine and histidine, and used successfully as the sole source of nitrogen. One conspicuous exception lies in the inability of my two avian strains to utilize alanine; they deaminize leucine readily however. The tubercle bacillus is able to thrive with alanine, leucine and histidine as sources of nitrogen, but not with tryptophane or phenylalanine. It might be thought that deaminization of these compounds did occur, but that growth did not proceed because the resultant product was toxic. As you know, one theory accounts for the stoppage of bacterial growth in old cultures on the ground that a high concentration of toxic products has been reached, for example from the tyrosine of the medium, which is nothing more than alanine with one hydrogen atom replaced by a molecule of carbolic acid, or from phenyl alanine where the replacement is with benzene. However, such reasoning cannot explain the failure of the tubercle bacillus to grow when phenyl alanine is supplied as the sole source of nitrogen, for when alanine is supplied in addition growth does occur.

As regards the utilization of the carbon in amino acids for structural purposes, it may be said that tubercle bacilli are unable to synthesize all the carbonaceous portion of their body substance from the material left after the abstraction of nitrogen. They need something else, best glycerol. Leprosy bacilli can slowly build up their organic body substance, all of it, from alanine or leucine. Frog, fish and turtle bacilli do the same thing a little better, but seem unable in general to split the

ring compounds, such as tyrosine, tryptophane and histidine. The same statement may be made of the various grass bacilli, except that they do seem to split the imidazol ring of histidine. I am in this case, of course, taking it for granted that growth on histidine, as sole source of both carbon and nitrogen, means a splitting of the ring, as, naturally, proteins cannot be built up simply from piled up histidine molecules, and we have no evidence that bacteria take the straight chain for structural purposes—I grant they may oxidize it off—and throw the ring away. No organism of the group seems able to utilize tryptophane or tyrosine as sole source of both nitrogen and carbon. Inasmuch as some of the saprophytic acid-fasts are able to thrive on tryptophane and phenyl alanine, when glycerol is supplied as an additional source of carbon, I assume that the failure to grow on the ring compounds without glycerol indicates the inability of the organisms to split the indol and benzene rings.

As regards the utilization of nitrogen in other forms, I have found that propionamide is readily deaminized by all organisms of the group, that ammonia is a sufficient source of nitrogen for all members of the group, that creatinine, which contains imino, but not amino nitrogen, is used for nitrogen fairly readily by smegma and grass bacilli, somewhat sparingly by frog, fish and turtle bacilli, less readily still by leprosy bacilli, and by tubercle bacilli, as far as I can detect, not at all. Urea, which may be thought of as an amino acid amide, is used readily by all forms except tubercle bacilli, and turtle and fish bacilli. The ready use by leprosy bacilli is noteworthy.

No study of nutrition nowadays would be complete without a consideration of vitamines. They have been described as favorable to the growth of a variety of bacteria by several investigators. To be brief, I have not been able to demonstrate any growth increase with tubercle bacilli or other acid-fast organisms following the addition of certain market preparations of water-soluble (yeast) vitamine, or at least any increase which could not be attributed to the presence of certain useful amino acids, any increase which could not be duplicated by the addition of an "aminoid" mixture in which no vitamine constituency was supposed to be present at all. On the other hand, I have been able to prove that certain amino acids are useful *per se*. You are doubtless familiar with the work of Osborne and Mendel and others, who have shown that in mammalian metabolism lysine and tryptophane are necessary for growth and maintenance. I have been able to show that

lysine, while not necessary to the growth of the tubercle bacillus, is at least desirable, for when you take two proteins, otherwise fairly similar, the one, edestin, containing lysine, the other, gliadin, being practically lacking in this substance, and prepare peptones from these and grow the tubercle bacillus upon them, you will find that the better growth occurs on edestin, the one with the amino acid lysine.

In conclusion, let me recapitulate the problems that I am considering, the study of which is being facilitated by a grant from this association. First, there is the type of nucleic acid present: what are its pyrimidine bases and what is its carbohydrate? Second, what sorts of protein are present in the tubercle bacillus? Then there is the subject of metabolism. How is phosphorus used? In the nitrogen metabolism how are amino acids broken up and what is the bearing of this breakdown on the reaction change of the medium? What happens to the cyclic amino acids, those substances from which physiologically active compounds are produced so readily? And lastly, a problem I have not yet touched: what is the rôle of glycerol in synthesis? This is a large order, and yet it touches but a few of the problems we must clear up for a moderately complete knowledge of the life history of the tubercle bacillus.

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THE NUTRITION OF ACID-FAST BACTERIA

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Ever since the possibility of confusion has existed, those microorganisms which stain with more or less difficulty with the common aniline dyes, but which, once stained, retain their color tenaciously against acid and alcohol, have been considered as a biological division. About forty types, described at various times, have been herded together in bacteriological classification, largely on the basis of this single property, and spoken of as the "acid-fast" group, in distinction from such groups as the "colon-typhoid" group, the "mucosus capsulatus" group, and so on. Some of these are certainly identical, while others have been considered by many investigators as mutually transmutable, perhaps derived originally from a common acid-fast ancestor. More conservative writers (1), (2), (3) doubt this transmutability, however, and prefer to distinguish two main types, pathogenic and nonpathogenic, with several subgroupings within the two main divisions, based on habitat.

It would seemingly not be justifiable to classify these bacteria on the basis of that single attribute, unless it were a fundamental one not possessed by other bacteria and maintained always under given conditions by the ones in question. Reduced to concrete terms the propriety of placing them in a single group, since it cannot be done on the basis of virulence, depends, in the opinion of the author, upon the answer to such questions as these: Do they have in common any of the recognized chemical carriers of specificity? Particularly, do they possess common or closely related proteins not included in the bodies of other bacteria?—protein being generally considered the carrier of hereditary characteristics (4). Do they become non-acid-fast under any conditions and breed true in the new state? Can any of the bacteria outside the group be made to take on this property of acid-fastness? In summary, is acid-fastness a manifestation of any biological individuality?

[1] Aided by a grant from the National Tuberculosis Association.

Now the nature and individuality of the proteins present in the protoplasm of different bacteria cannot be readily revealed by the present coarse methods of chemical analysis. Fortunately, however, we have most delicate tests of another kind, the so called biological ones, mysterious in their working, but none the less accurate, which permit a sharp differentiation of proteins. And on the basis of such tests we do have reason for believing that certain of the acid-fast bacteria possess common proteins not present in the bodies of certain other bacteria. The information is incomplete and will require much extension to furnish the basis for the formulation of a law, but it seems definite as far as it goes. Krause and Baldwin (5), who have reviewed the investigations of others, find in their own experience that guinea pigs sensitized with any of the following extracts: human type tubercle, butter, leprosy, timothy, milk, dung and smegma bacilli, will react to their proper antigen or to that of any of the others, but that none of the animals so sensitized will react to B. subtilis emulsion, nor will subtilis sensitized pigs react to the acid-fast preparations. On the other hand, Urbain and Fried (6) and others have noted a biological relationship between the tubercle bacillus and the diphtheria bacillus, in obtaining positive complement fixation with either bacterium as antigen in disease caused by the other. A situation of multiple antigen, somewhat akin to that discussed by Much, is quite naturally suggested.

Acid-fastness is most commonly explained as a function of the high wax content of these organisms. Yet it is far from likely that the wax itself is a carrier of any specificity (4). It is probably only associated with it, in which case it would be dependent upon some more significant underlying factor of nutrition. It should be mentioned that some non-acid-resistant saprophytic bacteria have been made to acquire acid-fastness by growth upon lipoid-rich media (7), and acid-fast ones to become non-acid-fast by cultivation upon media not readily adapted to the synthesis of fat (8).

Now a certain amount of specificity is unquestionably determined by the presence within a cell of a high concentration of a given enzyme (4). That statement altogether begs the question whether enzymes are proteins or not. A pancreas cell differs from a liver cell in the marked ability of its expressed juice to break down protein subjected to its destructive influence. It contains a high concentration of what we call tryptic ferment. A liver cell apparently differs from one of the intestinal mucosa in its faculty for working up the ammonia elaborated

in the gastrointestinal tract into the innocuous substance, urea. Crudely expressed, these cells differ in one respect in that they work with different tools, more or less specific for each.

Bacteria, too, differ among themselves in the same way, and several good but incomplete classifications are based on the differences observed. The experiments recorded below represent a study of the similarities and differences in the action of the acid-fast bacilli upon some of the common food stuffs provided for them in nature. The attempt was made to learn whether they possess common nutritional properties as well as the compositional one expressed by the phenomenon of acid-fastness. No effort has thus far been made to trace their development from these foods. They were furnished with simple diets containing all the necessary elements for the construction of protoplasm, N, C, H, O, P, S, K, Na, Ca, Mg, and Cl; and they grew or died, according as they did or did not possess the tools for obtaining their livelihood from the materials in the form provided.

Nitrogen was furnished in certain well defined protein decomposition products, in amino, amide, amine and imino form. Carbon was supplied in the form of amino acids or certain alcohols and acids. P, S, K, Na, Ca, Mg, and Cl were kept constant in all experiments, the media containing 0.5 per cent NaCl, $\frac{N}{50}$ KH_2PO_4 and traces of $MgSO_4$ and $CaCl_2$. In addition, 2 per cent purified agar was used in all experiments except those with tubercle bacilli (human and bovine types). This may be considered an inert substance. Cultures were run in quadruplicate.

In the tables below growth is recorded by an arbitrary system of pluses, one plus meaning distinct but not heavy growth, two pluses, moderately heavy, and three pluses, profuse. Human and bovine type tubercle bacilli were grown for one month, and all others for two weeks. All were grown at 37°C. except the frog, fish and turtle bacilli, which were kept at 25°.

The isolation histories of the bacteria used were not known in all cases. H37 and R1 are old cultures of tubercle bacilli of the human type, isolated from cases of pulmonary tuberculosis seventeen and thirty years ago, respectively, the first of relatively high, the second of decidedly low virulence. H102 is a human type bacillus isolated from sputum in 1920. B1 is a bovine type bacillus isolated from a cow's lung in 1902. A1 is an avian bacillus isolated from a chicken in a California epidemic in 1905. The timothy bacillus and frog bacillus are descendants of

TABLE 1

*Growth on amino acids + 5 per cent glycerol**

AMINO ACID	H_{37}	H_{102}	R_1	B_1	A_1	A_2	LEPRA HARDY	LEPRA DUVAL	FROG	TURTLE	FISH	SMEGMA N. W.	SMEGMA S. L.	GRASS	GRASS III	GRASS IV	MIST	TIMOTHY
d-l Alanine	++	+	+	++	0	0	++	+++	+++	++	++	+++	+++	++	+++	+++	++	++
l-Alanine†	++			++	0	0	++	++	++	++	++	+++	+++	+++	+++	+++	+++	++
Leucine	+			+	++		++	++	++	++	++			++	++			+
Histidine	+				+	+	++	++	++	+	+							
Tryptophane	0			0	0			0	0	+	0		+	+	+			+
Phenyl alanine	0			0	+	+	0	0	+	0	0	+	+	0	+	0	0	0
Tyrosine	0	0	0	0				0	+				0					0

* In addition to $\frac{M}{10}$ concentration of the amino acid (except in the case of tyrosine, which was used in saturated solution) and the glycerol, each medium contained 0.5 per cent NaCl, M/50 KH_2PO_4, traces of $CaCl_2$ and $MgSO_4$, and was adjusted with NaOH to P_H 7.0.

† L-alanine was prepared by the method of Emil Fischer by benzoylating d-l alanine, preparing the brucine salts of the benzoylated compound, separating these by fractional crystallization, removing the brucine by alkali, and further purifying the l-compound through the strychnine salt. The strychnine was then removed as usual with alkali, and the resulting l-benzoyl alanine hydrolyzed with 20 per cent HCl to free the alanine. The possible racemizing effect of this last procedure cannot be ignored. The l-benzoyl alanine prepared as above gave approximately the same specific rotation as that observed by Fischer; the l-alanine obtained on its hydrolysis, very little rotation, if any. However, according to Fischer, optically active alanine has a very slight specific rotation, not nearly as great as its benzoyl modification. Under these circumstances I am not sure that I was using l-alanine free from the d-variety. It is usually stated that only the d-compound is biologically utilizable, although the evidence for this is incomplete. It will be seen in table 1 that the substance I used was readily utilized as a source of N when glycerol was present, and as a source of both N and C (table 2) when glycerol was absent, growth being not quite so good as with the synthetic alanine, which unquestionably contained 50 per cent of the d-variety.

TABLE 2

*Growth on amino acids without glycerol**

AMINO ACID	H_{37}	H_{62}	R_1	B_1	A_1	A_2	LEPRA HARDY	LEPRA DUVAL	FROG	TURTLE	FISH	SMEGMA N. W.	SMEGMA S. L.	GRASS	GRASS III	GRASS IV	MIST	TIMOTHY
d-l Alanine	0	0	0	0	0	0	+	+	++	++	++	++	++	++	++	++	++	++
l-Alanine					0	0	0	0	+	+	+	+	+	+	+	+	+	+
Leucine	0			0	0			+	++	++	++		+		+			+
Histidine	0				0	0	0	0	0	0	0	++	++	+	++	++	+	0
Tryptophane					0			0	0	0	0		0	0	0			0
Phenyl alanine					0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tyrosine	0	0	0	0	0			0	+				0					0

* See foot notes to table 1.

TABLE 3

Growth on amino acids + glycerol. Effect of phenyl ring*

AMINO ACID	H_{37}	B_1	A_1	A_2	LEPRA HARDY	LEPRA DUVAL	FROG	TURTLE	FISH	SMEGMA N. W.	SMEGMA S. L.	GRASS	GRASS III	GRASS IV	MIST	TIMOTHY
$\frac{M}{10}$ alanine	++	++	0	0	++	+++	+++	++	++	+++	+++	++	+++	+++	++	++
$\frac{M}{15}$ phenyl alanine	0	0	+	+	0	0	+	0	0	+	+	0	+	0	0	0
$\frac{M}{30}$ phenyl alanine + $\frac{M}{20}$ alanine	+	+	0	0	0	0	+	+	+	++	++	+	++	++	+	+

* See foot note to table 1.

TABLE 4

Growth on amino, amide, amine, imino, and ammonia N (the medium contains glycerol)*

NITROGEN	H37	H102	R1	B1	A1	A2	LEPRA HARDY	LEPRA DUVAY	FROG	TURTLE	FISH	SMEGMA N. W.	SMEGMA S. L.	GRASS	GRASS III	GRASS IV	MIST	TIMOTHY
Alanine	++	+	+	++	0	0	++	+++	+++	++	++	+++	+++	++	+++	+++	++	++
Propionamide	+	++	+	+	+++	+	+	+	+	0	+	++	++	++	+	+	++	+
Ethyl amine (HCl)	0	0	0	0	0			0	+	0	0		0	0	++	++	0	0
Ammonia (HCl)	++	++	+	++	++	++		+	+	+	+		+	+			+	+
Creatinin	0			0	0	0	+	0	+	+	+	+	++	++	+++	+++	++	++
Urea	0			0	0		++	++	+	0	0	++	++	++	++	++	++	++

* See foot note to table 1.

TABLE 5

*Growth on ammonia + various substances containing carbon**

NUTRIMENT	H37	H102	R1	B1	A1	A2	LEPRA HARDY	LEPRA DUVAL	FROG	TURTLE	FISH	SMEGMA N. W.	SMEGMA S. L.	GRASS	GRASS III	GRASS IV	MIST	TIMOTHY
$NH_4Cl + C_2H_5OH$	0			0	0	0		0	+	0	0		+	+	+	+	+	+
$NH_4Cl + CH_3OH$	0			0				0	0	0	0		0	0	0	0	0	0
NH_4Cl + glycerol	++	++	+	++	++	++		+	+	+	+		+	+			+	+
NH_4Cl + glycol	0			0	0			0	0	0	0		0	0			0	0
NH_4 propionate	0	0	0		0	0	0	0	++	++	0	++	++	++	++	++	++	++
NH_4 acetate					0	0		0	0	0	0		0	0			0	0
NH_4 lactate	0	0	0	0	0	0	0	+	++	0	0	++	++	++	++	++	++	+
NH_4 glycollate					0	0	0	0	0	0	0	0	0	0	0	.0	0	0
NH_4 pyruvate	0	0	0	0	+	0	++	+	++	+	++	+	++	++	++	++	++	++
NH_4 formate							0	0	0	0	0		+	0	+	+	+	+
NH_4 oxalate					0	0		0	+	+	+		+	+			+	+

* See foot note to table 1.

strains isolated by Moeller in Berlin about 1904. The turtle bacillus culture is the same as that brought to this country by Friedmann in 1913. The others are stock strains of some years standing from the American Museum of Natural History, and the Hygienic Laboratory and the Department of Bacteriology of the University of Chicago, for which exact histories were not available. All were acid-fast and remained so on all media used.

SUMMARY OF RESULTS

In table 1 growth with a number of amino acids as sole source of N and with glycerol as an additional source of C is recorded. These being all α-amino acids, the N has the same relationship to the rest of the molecule in each case: that one expressed by the formula R. CH. NH_2. COOH. R is either CH_3, as in the case of alanine, or CH_3 with one H atom replaced by another group. In all except leucine the replacing group is a ring structure. Table 2 records the availability of the factor R in its various forms for building the entire carbonaceous portion of the bacterial body, glycerol being left out of the medium, the remainder of the amino acid after the extraction of the amino group constituting the sole supply of C.

Summarizing the results obtained, we note that the amino group is utilizable by all bacteria as their sole source of N. With all bacteria used except the avian bacillus, alanine served readily as a source of N. Why alanine could not be used by avian bacilli, when isopropyl alanine (leucine) and imidazol alanine (histidine) were utilized, awaits later explanation. It is easier to explain why growth was relatively feeble when tryptophane (indol alanine), phenylalanine and tryosine (oxyphenylalanine) were used. These compounds contain the benzene nucleus, which in small amounts is toxic to bacteria. With each atom of N withdrawn, in the case of phenylalanine, for instance, a molecule of phenyl lactic or phenyl pyruvic acid is left as a possible interferent with growth, even when an adequate supply of C is available from another source, glycerol.

This factor of toxicity may be further studied in table 3. Three media were used for this experiment. In each case the same amount of N was present. In one medium was $\frac{M}{10}$ alanine, in another $\frac{M}{10}$ phenylalanine, and in the third $\frac{M}{20}$ alanine plus $\frac{M}{20}$ phenyl alanine, in addition in each case to 5 per cent glycerol. It will be seen that with the excep-

tion of the avian bacillus, for which an inverse relation obtains, as noted above, all bacteria grew well with alanine as a sole source of N, sparsely or not at all with phenylalanine, and that in the case of human and bovine type tubercle bacilli, turtle and fish bacilli, smegma and various types of grass bacilli, growth was improved by replacing half of the phenylalanine with alanine, but that such substitution did not restore growth to the level obtained with alanine in the absence of its phenyl modification. It is of course conceivable that bacteria use a different deaminizing enzyme for each amino acid, and that those studied here are better equipped with the one for alanine than that for phenylalanine, but it seems more probable that the factor concerned in the decreased growth with the latter is the toxicity of the compound left after the deaminization.

Table 2 shows the availability of the carbon residue left after deaminization. This residue seems useless to the tubercle bacillus in all cases, this organism being strongly glycerophilic. The leprosy bacillus on the other hand possesses the necessary tools for working up the residue from alanine and leucine, at least, into its own substance and maintaining itself on these residues as the sole source of its carbon. It is unable however to use the deaminized residue from histidine (from which it is able to abstract N: see table 1). Frog, turtle and fish bacilli behave in an entirely similar manner. In all probability we are dealing here with an inability to split the imid-azol ring of histidine into the simple groups from which synthesis of the necessary variety of bacterial building stones can start.

Smegma bacilli are better equipped for coping with an unfavorable environment, in that they possess the mechanism for deriving their entire carbonaceous nutriment from the imid-azol-propionic arrangement of C atoms, in all probability splitting the imid-azol ring. The same statement holds for most of the grass bacilli, the dung bacillus being considered a grass bacillus. Smegma and grass bacilli are however unable to get the necessary nourishment from the carbon residue of tryptophane, although, as table 1 shows, they are able to deaminize it. This means, either that they are unable to split up the indol-propionic grouping, or that the latter is too toxic. Probably the former possibility is the correct explanation, in view of the fact that some growth does take place when glycerol is supplied as an additional source of C, none at all occurring on tryptophane alone.

In table 4 a comparison is made of the utilization of N in various forms, amino, amide, amine, imino, and ammonia. In each case an adequate supply of C was presented in glycerol. Summarizing, we note that the amino group of alanine is readily used by everything except avian bacilli. The acid amide group of propionamide is used by all bacteria except the turtle bacillus. The ammonia grouping serves as the sole source of N for any of them, and it is probably chiefly by conversion into this form that the N of any chemical grouping is used. Creatinine is not used by tubercle bacilli, may or may not be used by leprosy bacilli, but is susceptible to the action of the bacilli of cold blooded animals, the smegma bacilli, and the various grass bacilli. It is an example of nitrogen in the imino grouping, vulnerable apparently to the above named group.

Urea, which may be looked upon as the diamide of carbonic acid or as the amide of carbamic acid, is not so readily utilized as might be expected. Readily available to leprosy bacilli, smegma bacilli, and all the grass bacilli, its N cannot be used by human, bovine or avian type, tubercle bacilli or by any of the bacilli of cold blooded animals except the frog bacillus.

Of all the bacteria used, only three possessed the power to use the N of ethyl amine. Curiously enough these were of different groups, two of them being grass bacilli, the other the frog bacillus. Tubercle bacilli were unable to utilize it. This experiment was repeated several times in view of the fact that two years ago the author (9) reported utilization of amine N by tubercle bacilli. As prepared, the amine used at that time must have contained some ammonium chloride, enough to account for growth of the tubercle bacillus. The product used in the experiments here recorded was ammonia-free, and it can be definitely stated that H37, B1, and A1 do not use this compound. In general the amines are stable compounds in the laboratory. The remarkable thing is not that so many bacteria failed to use ethyl amine nitrogen, but that some were able to do so. The frog bacillus and grass III and IV obviously possessed something lacking in the other bacilli, a mechanism for prying the NH_2 group off from a primary amine. No explanation can be given of the difference in the behavior of the frog bacillus and its supposed relatives, the turtle and fish bacilli, and in that of grass III and IV and the other grass bacilli. It was sharp and unquestionable, however, serving to show how complicated the problem of differentiating bacteria on a chemical basis may be. As a point of interest it

may be mentioned that a culture of the frog bacillus contaminated with B. subtilis was readily cleared of the contamination by growth upon an amine medium, in which ethyl amine was the sole source of both N and C, B. subtilis being unable to use the amine.

The action of the frog bacillus was further examined in the following experiment. Fifteen bottles of a medium containing ethyl amine hydrochloride as the entire supply of N and C, after serving for the growth of the frog bacillus for one month, were distilled, and the distillate was treated with ammonia and Lugol's solution. No crystals were formed, but a marked odor of iodoform developed, indicating the production by the frog bacillus of a certain amount of either ethyl alcohol or acetaldehyde in the medium. A control of fifteen bottles inoculated at the same time with the grass bacillus, which did not utilize this compound, was subjected to the same treatment and failed to give the test.

In table 5 is indicated the utilization of C in various forms, from which only empirical deductions can be drawn at the present time. Glycerol is readily used by all members of the group. Glycol, its homologue with one less carbon atom, is not utilized by any. Ethyl alcohol has a limited utilization, serving as a sufficient source of carbon to the frog bacillus, smegma bacillus and grass bacilli, and perhaps some of the others not tried. Methyl alcohol, its homologue with one less carbon atom, is unutilizable. So much for alcohols. Propionic acid served as a complete source of carbon for frog, turtle, smegma and grass bacilli, but not for tubercle, leprosy or fish bacilli. Its homologue with one less C atom, acetic acid, was unutilizable. Lactic acid was similar in its nutritive value, except that the leprosy bacillus did use it and the turtle bacillus did not. Its homologue with one less carbon atom, glycollic acid, was unutilizable. Pyruvic acid sufficed as sole source of carbon for all bacteria except human and bovine type tubercle bacilli and one of the avian strains. Its homologue, glyoxylic acid, was unfortunately not available for investigation. Most remarkably, for some bacteria formic and oxalic acids could serve as the sole source of carbon. These substances contain only H_2 as available energy; unknown reduction processes go on to render their carbon utilizable.

In general, it will be noted from the above summary that the three carbon atom acids are utilizable for synthesis, and the two carbon ones not. It is to be noted that the alcohol related to the three carbon atom acids in sugar metabolism, equivalent to lactic acid minus carbon dioxide, namely, ethyl alcohol, is utilizable, while the alcohol similarly

related to the two carbon acids, methyl alcohol, is unutilizable. The usefulness of glycerol and nonutility of glycol for this group of bacteria is no new observation, it having been recorded years ago by Proskauer and Beck (10).

DISCUSSION

The protocols above record the results of some four hundred and fifty culture experiments, or about eighteen hundred cultures, since each experiment was carried out in quadruplicate. Naturally, in a series of that size many discrepancies occur. Space would not permit a separate discussion of all of these, nor would such discussion, indeed, be profitable. We cannot ignore the fact that bacteria as living things possess a certain variability not encountered in standard, dead chemical reagents. I have seen a chromogenic strain of grass bacillus run pure white through three weeks on one transplant in quadruplicate and return to a bright yellow in the transplant from this to the same medium. It is no cause for wonder that their chemical action on food stuffs is not always as expected.

In spite of this fact and in spite of the fact that the above tables are obviously incomplete, various strains being added or withdrawn in the course of the investigation, perhaps enough broad generalizations may be drawn from them and the summaries given in the text, to furnish a start on classification, subject always to revision. Furthermore, if this classification is to be useful in a practical way, it must recognize the point of view of parasitism as against avirulence.

One of the characteristics of saprophytes as seen in the uncontrolled conditions of nature, is their ability to break down very complex into simple compounds, from which new life is constructed, the familiar organic decay, which constitutes an arc in the great carbon and nitrogen cycles. Complex chemical groupings such as the benzene and indol rings are torn to pieces, the imino grouping is vigorously attacked by saturation with water, and ammonia is pried loose from stable things like amines. In a general way parasites are more helpless, and more likely to thrive on nutriment a portion of which at least is predigested, and many of them are prone to leave untouched difficultly digestible morsels like the aromatic rings. Numerous exceptions to these propositions can of course be cited, but they approximate the truth closely enough to be useful.

It will be noted in table 5 that the alcohols other than glycerol, and the organic acids used, are insufficient sources of carbon for the tubercle bacilli. They must have something more, preferably glycerol. They are thus at once set apart from the rest of this heterogeneous group, which use these substances to a varying degree. Turning now to some of those characteristics of saprophytism just mentioned, we note that the complex imid-azol-propionic structure (histidine) is utilizable by smegma and grass bacilli as a source of carbon and useless to the others. Something like the same separation can be made on the basis of the utilization of the nitrogen of the complex tryptophane (table 1). Utilization of the creatinin imino nitrogen and abstraction of nitrogen from urea again place the smegma bacilli with the grass bacilli and both of them apart from the tubercle bacilli. By exclusion of these two extremes, we have left the leprosy bacilli, provisionally so called, with due recognition of their insecure etiological status, and the bacilli of cold blooded animals. In their utilization of the carbonaceous portions of d-l alanine and leucine, these behave somewhat alike and more like the grass and smegma bacilli than like the tubercle bacilli. In their less general utilization of propionic and lactic acid and ethyl alcohol as sources of carbon, they act more like the tubercle bacilli. In their action on creatinin they are again more like the grass bacilli. In their failure to use the carbonaceous part of histidine they behave similarly and resemble more the tubercle bacilli.

From the above considerations it will at least be seen that the tubercle bacilli on the one hand and the smegma and grass bacilli on the other, fall rather readily into separate nutritional groups. The lepra and cold blooded animal bacilli have many points of difference from both of these and certainly a few nutritional factors in common. Tentatively they can perhaps be considered together as a third group. Finally it should be added that the distinction between human and bovine type tubercle bacilli has so far proved too subtle to be drawn on a nutritional basis.

REFERENCES

(1) WEBER AND TAUTE: Tuberk-Arb. a.d. kaiserl. Gesundheitsamt, 1905.
(2) GOTTSTEIN: Hyg. Rundschau, 1905, xv, 281.
(3) KRAUSE: Jour. Outdoor Life, 1920, xvii, 260.
(4) LEVENE: Jour. Amer. Chem. Soc., 1917, xxxix, 828.
(5) KRAUSE AND BALDWIN: Trans. 9th Annual Meeting, Nat. Assn. Study & Prev. Tuberc., 1913.
(6) URBAIN AND FRIED: Ann. de l'Inst. Pasteur, 1921, xxxv, 294.
(7) BIENSTOCK AND GOTTSTEIN: Fortschr. d. Med., 1886, iv, 193.
(8) WHERRY: Jour. Inf. Dis., 1913, xiii, 144.
(9) LONG: Amer. Rev. Tuberc., 1919, iii, 86.
(10) PROSKAUER AND BECK: Zeitschr. f. Hyg., 1894, xviii, 128.
(11) FISCHER: Untersuchungen über Aminosauren, Polypeptide und Proteine, Berlin, 1906, p. 90.

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THE PRESENT STATUS OF THE PROBLEMS OF ANAPHYLAXIS

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The literature of anaphylaxis and related topics, and the number of unsettled problems, are both too vast to permit of an exhaustive review within the scope contemplated by these PHYSIOLOGICAL REVIEWS, even if there were not already numerous excellent and adequate compilations of this sort in available literature (1). In order to avoid covering again too much of the ground already thoroughly surveyed by others, this review will limit itself to discussing what seem to be the most essential problems in the light of the more recent contributions.

DEFINITION AND LIMITATION OF THE TERM "ANAPHYLAXIS"

Much unnecessary work and many misleading statements would have been avoided in the past if more attention had been given to these essential points. Not all sudden attacks of dyspnea and convulsions in injected guinea pigs, or of cardiac syncope or cutaneous irritation in man, are manifestations of anaphylaxis, or are even related to this condition beyond in a greater or less similarity of these particular symptoms. Even distention of the lungs in guinea pigs, circulatory disturbances in rabbits, or splanchnic congestion in dogs, following intravascular injections of foreign materials, are not necessarily proof of a true anaphylactic reaction, for each and all may be produced by materials or under conditions that surely do not involve the same processes that are responsible for the reaction which occurs when a small quantity of an otherwise harmless protein is injected into an animal that has been previously prepared at a sufficiently great interval of time, by injection of the same protein. We must grant that this last is true anaphylaxis, and must demand that the term anaphylaxis be limited to conditions that result from a similar mechanism within the body of the reacting animal. At least the following criteria must be met:

1. The observed toxicity of the injected material must depend upon the sensitization of the animal; i.e., the substance must not produce similar symptoms in non-sensitized animals.

2. The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being therefore the same for all antigens with the same test animal, but differing characteristically with each species of animal.

3. It should be possible to demonstrate passive sensitization with the serum of sensitized animals.

4. It should be possible to demonstrate typical reactions in the virgin guinea pig uterus strip.

5. It should be possible to demonstrate amelioration or prevention of the bronchial spasm in guinea pigs by proper use of atropin and epinephrin.

6. The possibility that the observed symptoms are caused by capillary thrombosis or embolism must be excluded.

7. After recovery from anaphylactic shock there should be exhibited a condition of desensitization under proper conditions.

We know that the foregoing conditions are fulfilled in the typical anaphylactic reaction with soluble protein antigens. Reactions produced by substances not coming within this class cannot properly be considered as true anaphylactic reactions unless they can be demonstrated to exhibit the same phenomena. The term allergy is broad enough to cover all those manifestations of altered reactivity and to indicate their relationship to one particular form of allergy, anaphylaxis.

An important series of papers by Karsner and Hanzlik (2) demonstrates the common sources of error in much of the published work on anaphylaxis, and especially on the supposed formation of anaphylatoxin in vitro by other than antigen-antibody reactions. It will be recalled that Friedberger, Bordet, Novy and DeKruif, and others, have observed symptoms considered to be characteristic of anaphylaxis following intravenous injection into animals of serum that has been acted upon by colloids, such as agar and inulin, or suspensions, such as kaolin or starch, or after injection of these same colloids or suspensions. Likewise, anaphylactic shock is said to have followed intravenous injections of acacia, arsphenamine and many other substances used in human therapeutics. In most of the animal experimentation the proof of the anaphylactic character of the reaction has rested not only on symptomatology but also on the presence of apparently typical bronchial spasm with persistent pulmonary distention in guinea pigs, or fall in blood pressure with splanchnic congestion in dogs.

Karsner and Hanzlik show that agar and numerous other substances produce "anaphylactoid" symptoms after intravenous injection, by virtue of their causing occlusion of the pulmonary capillaries by thrombosis or by agglutinated corpuscles or platelets. With such capillary obstruction, which is demonstrated both by finding the emboli and by the stoppage of perfusion fluids injected into the pulmonary vessels, there may occur a marked bronchial constriction which causes asphyxia and permanent distention of the lungs. This bronchial constriction, which has commonly been interpreted as proof of a true anaphylactic shock, differs from that of true anaphylaxis in not depending on a contraction of the bronchial musculature, which they contend is shown by the failure of epinephrin and atropin to influence the respiratory effects of agar in the same way that they control anaphylactic bronchial spasm. "On the basis of the results obtained with atropin and epinephrin, the mechanism of the action of agar and similar agents bears no relationship to true anaphylaxis or anaphylactic shock." How the "passive broncho-constriction" of agar intoxication is produced is not altogether satisfactorily explained.

> The agar forms thrombi which block the small pulmonary vessels, surrounding the bronchioles, which in turn are compressed by the *vis a tergo* of the pulmonary flow from the right heart of the intact animal, and of the perfusion fluid in surviving lungs. In the perfused lung the flow of perfusion fluid stops. The compressed bronchioles are sufficiently narrowed in caliber to prevent the escape of the air forced in by the violent efforts of inspiratory dyspnoea of the intact animal and the artificial respiration of the perfused lungs. In other words there is a passive broncho-constriction (really a compression of the bronchi), and as a result permanent inflation of the lung. . . . . It is conceivable that additional broncho-constriction is superimposed as a result of central asphyxial stimulation of the vagus (parasympathetic) on the passive constriction primarily brought about.

The capacity of agar to produce agglutination of red corpuscles is very marked. Concentrations as low as 0.001 per cent cause agglutination in vitro, while the corresponding figures for other colloids (e.g., acacia, 1.0; althea, 0.08; collargol, 0.005; gelatin, 0.05; nuclein, 0.5; beef serum, 0.005 per cent) indicate their capacity to cause anaphylactoid symptoms when injected intravenously. Therefore it is not surprising that on intravenous injection "the quantity of agar necessary to produce profound effects and even death, is remarkably small, namely, about 0.014 to 0.05 mgm. per gram of body weight, or about 0.014 to 0.025 per cent concentration of agar in the blood."

Perhaps it should be added that "no emboli, thrombi or conglutinated corpuscles were demonstrable in perfused or intact lungs of anaphylactic shock, after peptone, certain serums and histamine. All of these agents were also active smooth muscle stimulants in other regions besides bronchi, namely uterus and intestine."

With the above facts established it must be recognized that much of the work published and interpreted as anaphylaxis deals rather with anaphylactoid phenomena. In working with vegetable proteins, the danger of confusion of capillary embolism is particularly marked, since many plant products are powerfully hemagglutinative, especially extracts from the leguminous seeds, while many vegetable proteins are thrown out of solution by serum.[1] I have, therefore, usually selected the intraperitoneal route with the view of avoiding these sources of error. Karsner and Hanzlik have not investigated the amount of obstruction to pulmonary capillaries that may follow intraperitoneal injection of foreign colloids. Presumably it is much less, and perhaps entirely absent, but they do mention some slight pulmonary symptoms following subcutaneous injections of thromboplastic agents. Certainly in anaphylactic investigations, especially when the intravenous injection is used, the burden of proof of the nature of observed reactions lies heavily upon the investigator.

NATURE OF THE ANTIGENS (ANAPHYLACTOGENS)

It now seems to be generally accepted that true anaphylaxis represents an antigen-antibody reaction, but as yet there is not such complete agreement as to what the antibody is or as to what substances may behave as antigens in this particular reaction, i.e., anaphylactogens. There is no doubt that any soluble protein which can act as an antigen in other immunological reactions can act as an anaphylactogen, and the only soluble proteins as yet found not to be antigenic are those which are, from the chemical sense, incomplete proteins. As might be expected, such proteins as have a very small variety of amino acids are the chief examples of the non-antigenic proteins, notably the protamines and histones (3); also the commonly-occurring complexes of these with non-protein radicals, notably, the nucleoproteins (4) or hemoglobins (5). However, so complex and soluble a

[1] Undoubtedly the results obtained with the juice of *Cotyledon scheideckeri* by Kritchewsky (94) are merely anaphylactoid phenomena from a hemagglutinative and precipitating plant extract.

protein as gelatin seems to have absolutely no antigenic power, whether tested by anaphylaxis or by more sensitive methods, as shown conclusively by the careful work of Strain (6). As the chief known difference between gelatin and the proteins that do exhibit antigenic properties lies in an almost complete absence of tryptophane and tyrosine and a very low phenylalanine content, it would seem probable that it is this deficiency in aromatic amino acids that is responsible for the lack of antigenic capacity. Furthermore, there do exist proteins which lack one or more amino acids which are commonly present in typical proteins, yet which are strongly antigenic (e.g., zein from corn, which lacks glycine and tryptophane; gliadin of wheat and rye, or hordein of barley, which contain no glycine or lysine and very little arginine or histidine) (7). The fact that these three last proteins, which are so extremely poor in diamino acids, are potent, while the protamines which consist chiefly of diamino acids, are inert, suggests that these three diamino acids are not of importance in the anaphylactic activity of proteins, but since no protein is known which does not contain either histidine or arginine, we cannot prove this point as it seems to be proved for lysine.

Solubility of the protein is essential, since although insoluble proteins may eventually be brought into solution in the animal body, the process is too slow to bring about reactions, and also it is probably accompanied by disintegration of the protein molecule. Heat, of degrees that do not disintegrate the proteins, affects them only to the extent that it makes them insoluble. There are, however, only a few known proteins that are not made insoluble by heat, and these, except gelatin, are antigenic despite boiling, these being casein (8), ovomucoid (9), the so-called proteoses of plant seeds (10), beta-nucleoproteins (4) and perhaps the capsular substance of pneumococci (11). Compound proteins will produce anaphylaxis if they are soluble and if the protein constituents are themselves antigenic, but apparently not when the protein radical is a non-antigenic histon or protamin. Thus, alpha-nucleoproteins (4) and hemoglobin (5) are non-antigenic, while mucins (12), beta-nucleoproteins (4) and hemocyanin (13) are antigenic.

A suggestive observation on the requirements necessary for anaphylactogenic activity is furnished by Ten Broeck's observations (14) with the racemized proteins of Dakin. These proteins, presumably because of changes in structural configuration, are not attacked by the ordinary proteolytic enzymes, and likewise they do not exhibit anaphylactogenic properties. Landsteiner's (15) studies on the precipitin

reaction with azotized proteins have led him to the conclusion that the significance of the proteins for the usual immunity reactions, except for their immunizing capacity, does not depend so much upon their particular chemical structure as on their molecular size and the properties depending thereon. Such large molecular complexes may be able to react with antibodies even when incapable of engendering their formation.

The question whether anything less than an entire protein molecule can act as an anaphylactogen has been the subject of much investigation. Although I have always obtained negative results with numerous products of protein cleavage, nevertheless Fink (16), making in my laboratory an extensive study of proteose fractions obtained from coagulated egg white by hydrolysis with steam under pressure, did find evidence of a very slight anaphylactogenic property in those fragments that were precipitated at $\frac{3}{4}$ and complete saturation with ammonium sulphate, but not with fractions precipitated at $\frac{1}{4}$, $\frac{1}{3}$, $\frac{1}{2}$ and $\frac{2}{3}$ saturation. However, these reactions consisted only of slight symptoms even after intracardiac injections of 25 to 50 mgm. of proteose, and were of significance chiefly because similar symptoms were not produced by the other fractions and because positive precipitin and complement fixation reactions were obtained with these same two fractions only. This result is particularly surprising since it would be expected that the fractions precipitated by smaller concentrations of ammonium sulphate would represent larger molecules more closely resembling the intact protein molecule.

Fink gives an elaborate critical review of the published work on antigenic properties of the cleavage products of proteins, which indicates mainly that much of it is worthless, and that there is no conclusive evidence of anaphylactic or other immunological reactions having been produced with proteoses and peptones, beyond the very slight positive evidence that he has secured with his own carefully prepared materials. The obvious source of error in much of this work lies in the fact that "proteose" and "peptone" preparations generally are themselves active stimulators of smooth muscle and perhaps capillary poisons (Dale), in animals that are not sensitized, and hence observed symptoms can differ in sensitized and nonsensitized animals only quantitatively. They readily produce anaphylactoid manifestations on intravenous injection. Corroborating this interpretation of the supposed positive anaphylaxis reactions with proteoses is the fact that nearly all attempts to produce precipitins or complement-binding anti-

bodies with the same protein cleavage products have given negative results.

It is appalling to observe the amount of uncritical work done with unpurified, unanalyzed commercial "peptones," usually Witte's products. Such work can produce absolutely no usable evidence concerning most of the problems under investigation, since the source and composition of the material is entirely unknown to the investigator, and it has been repeatedly shown that Witte's peptone is a mixture of widely varying composition in different samples.

If no positive results can be obtained with most of the fractions of protein cleavage, including digestion mixtures of varying sources, but containing usually all the cleavage products from proteoses through peptones and polypeptids to amino acids, it is not easy to accept the statement that anaphylaxis can be produced by synthetic polypeptids, even one containing 14 molecules of leucine and glycine (Abderhalden, 17), still less the positive results of Zunz (18) with much simpler polypeptids (3–5 glycylglycine). These reactions were obtained after intravenous injections of the peptids into sensitized rabbits, and consisted of fall of blood pressure, increased rate of respiration and expulsion of feces and urine, but these reactions, slight as they are, were by no means constant, and occurred under very particular conditions, e.g., positive results with animals sensitized with six intraperitoneal injections at weekly intervals, but not in animals that received six subcutaneous injections at similar intervals. No other species of animals seems to have been tried, and it seems most improbable that the reactions observed would fulfil the requirements for proof laid down in the opening section of this review. Certainly, in view of the possible sources of such reactions independent of true anaphylactic shock, and the large number of negative results with peptones and digestion mixtures, the acceptance of the results yet obtained with synthetic polypeptids as proof of their antigenic activity will require much more convincing evidence than any that has yet been produced.

There still remains no satisfactory proof that anything except proteins can act as anaphylactogens. Even some definite antigens, whose protein nature is doubtful, are not anaphylactogens in the strict sense, namely, true soluble toxins and tuberculin, despite the fact that they do produce a certain type of hypersensitiveness (see Coca, 1). While many attempts have been made to establish the antigenic activity of lipoids by means of the precipitin and complement fixation reactions, the lack of solubility of the lipoids has made them unsuitable for ana-

phylaxis studies. Although a few studies with such material have been reported to give positive results, it is probable that these are all erroneous (see White, 19). Certainly as yet the antigenic capacity of lipoids has not been established.

On the other hand drugs and chemicals of many sorts have produced both local and systemic reactions that have been interpreted as anaphylaxis. Especially is this true of mercurials, salvarsan and other arsenicals, iodin and bromine and compounds thereof: also innumerable alkaloids (e.g., quinine, atropin) synthetic organic compounds (salicylates, antipyrine) and various vegetable oils, resins, etc. The evidence concerning the reactions produced by these substances has been thoroughly reviewed by Coca, who concludes that their anaphylactic nature has not been established. Wolff-Eisner has advanced the hypothesis that drug reactions may be produced by the blood or tissue proteins altered by the chemical so that they are as foreign proteins to the injected animal. As yet this hypothesis is unproved, although it is worth more extended and careful investigation than it has yet received. It is probable that many of the systemic reactions observed after intravenous injections of arsenicals and other substances are not anaphylactic but anaphylactoid, and dependent upon capillary thrombosis, agglutination emboli, or capillary toxicity, after the order of the results obtained by Karsner and Hanzlik. The failure to produce passive anaphylaxis with the serum of persons hypersensitive to drugs, the failure to secure convincing active sensitization in guinea pigs, the number of instances in which drug hypersensitiveness is exhibited on the first injection and those in which heredity is evidently responsible, and the numerous points of difference from true anaphylactic reactions, all make the identity of drug hypersensitivity with true antigen-antibody anaphylaxis, a matter of great improbability.

THE NATURE OF THE IMMUNE-BODY

Anaphylactin, sensibilizin, sensitizin

The demonstration that anaphylactic sensitization can be transferred to a normal animal by injection of blood or serum from a sensitized animal, has been generally accepted as establishing anaphylaxis as a typical antigen-antibody reaction. Doerr and Russ added important support to this view by showing that the potency of a serum in conferring passive sensitization varies in direct proportion to its content in precipitins. Zinsser states that "The principle that anaphylaxis

depended directly on the meeting of the antigen with its specific antibody has never been seriously questioned since that time." Three main questions have remained unsolved, however, namely: *a*, Is the sensitizing antibody the same as the precipitin or the complement-fixing antibody? *b*, Where does the reaction of antigen and antibody take place? *c*, How does this reaction produce the manifestations of anaphylactic shock?

THE RELATION OF PRECIPITIN TO THE SENSITIZING ANTIBODY

An attractive hypothesis is that there are two fundamental types of immunity reactions. One, having to do with substances which are essentially active poisons, neutralizes or inhibits their toxicity by direct chemical action (see Zinsser, 1). In this group come the antitoxins and the antibodies for enzymes, venoms, poisonous vegetable proteins (ricin, abrin, etc.) and various bacterial hematotoxins. It is to be noted that these active substances are all similar to one another in being classed as large colloidal aggregates, resembling proteins, but not yet identified as proteins. The other group of immunity reactions is concerned with defense against foreign proteins, whether toxic or non-toxic and whether in solution or aggregated into cells (bacteria, corpuscles, tissue cells). In all the reactions of this group we deal with processes that tend to alter the colloidal state of the foreign proteins, by making them larger aggregates (precipitation, agglutination) or smaller aggregates (proteolysis, hemolysis, bacteriolysis, cytolysis), and in each case the reaction consists of two separable steps, sensitization and reaction. It is tempting to accept the view, championed especially by Friedberger, that in this second group of reactions but one and the same antibody is concerned, and that all the reactions are essentially the same, differing merely in the method by which the reaction is demonstrated. There are, indeed, many facts capable of interpretation as supporting this hypothesis, but there are still other items that do not harmonize with it, notably the lack of constant quantitative relations between the different reactions produced by the same immune serum, and as yet it is neither established nor completely disproved.

Anaphylaxis obviously comes into the second class of "proteolytic" immune reactions, and the identity of the sensitizing antibody and the precipitin has been strongly supported. Several observers have found that the relative proportion of precipitin and sensitizing antibody runs parallel in immune sera (Friedberger, Doerr and Russ) and I have

myself observed, in studying the development of antibodies in animals immunized to vegetable proteins, that the precipitins appear together with the capacity to confer passive sensitization (20). Richard Weil (21) added much support to this contention by finding that the precipitate obtained when immune serum reacted with the specific protein, and washed free from the serum, conferred passive sensitization to the specific antigen when injected into guinea pigs, if the proportion of antiserum and antigen had been suitably selected in producing the precipitate. Mechanical carrying down of serum antibodies was excluded by appropriate experiments. Although it was found that if immune serum is heated at 70° for one-half hour it loses its precipitating power but not its capacity for passive sensitization, Weil did not consider this as evidence of a separation of precipitin and sensitizin, but interpreted it as indicating that only the thermostable combining (haptophore) group of the antibody is essential to passive sensitization. "The fact that the coexistence of antigen in the same fluid may inhibit the precipitating power of antibody, while only partially interfering with the sensitizing function, may explain the divergence in the literature between those who maintain that precipitin and sensitizin run parallel in immune serum and those who deny this relationship." The precipitates produced in the precipitin reaction contain both antigen and antibody, since they sensitize both actively and passively, and Weil believed that both antigen and precipitin can be separately extracted from such precipitates by suitable procedures. Although Coca (22) was unable to repeat Weil's experiment with washed precipitin-antigen precipitate, yet he did secure further evidence that precipitin and sensitizing antibody are identical.

Granting the identity of precipitin and sensitizing antibody, one must consider the relation of both to the complement fixing antibody, which numerous observers have maintained is identical with the precipitin (23). The assumption of identity of the sensitizin and the complement fixation antibody is the basis of Friedberger's classical experiments on the production of a poison, supposed by him to be responsible for anaphylaxis, by digestion of sensitized proteins with complement. This topic will receive consideration later.

THE SITE OF THE REACTION

The earlier view that anaphylaxis resulted from reaction of antigen and antibody in the circulating blood was shaken by the observation that in passive sensitization of guinea pigs there is a latent period of at

least four hours before the animal becomes sensitive, and that simultaneous injection of antigen and antiserum produce no effects. This fact is difficult to explain if antigen and antibody react in the circulating blood, but fits in well with the hypothesis that the latent period is required for the antibody to enter the cells and render them sensitive. The humoral reaction hypothesis was rendered untenable as a complete statement by the observation of Schultz that isolated segments of intestine from sensitized animals reacted to the specific antigen by contraction, and by the development and refinement of this principle especially by Dale and Weil. It has now been established that isolated non-striated muscle tissue, washed as free as possible from blood, will give strong contractions immediately it is brought into contact with even very dilute solutions of the specific antigen, and only with the specific antigen. The possibility that even the most careful washing out by perfusion methods may leave blood in some of the capillaries, especially emphasized by Larson and Bell (24), seems to be eliminated as an explanation of the positive results, both by quantitative considerations and by the observation of Weil (25) that uterine muscle of a passively sensitized guinea pig does not react to the antigen during the latent period (even if not perfused and full of blood) although at this time the blood in the uterus contains an abundance of antibodies. On the other hand a very much smaller dose of antiserum, even when followed by careful perfusion twenty-four hours later, has then rendered the uterine muscle highly reactive to the specific antigen.

Besredka (1), who was one of the earliest supporters of the "cellular theory" of anaphylaxis, believed that the reaction occurred in the cells of the central nervous system, but this localization is excluded by many experiments which show that anaphylactic reactions occur in tissues entirely disconnected from the central nervous system, as well as by the muscle strip experiments mentioned previously. Another form of evidence of the non-humoral nature of anaphylaxis is furnished by transfusion experiments (Manwaring, Pearce and Eisenbrey, Weil and others) which show that the blood of a sensitized animal can be replaced with the blood from a non-sensitized animal without impairing its sensitivity. This form of experiment is particularly convincing when passive sensitization is employed with serum of known sensitizing power, since then it is possible to know surely that the transfused animal cannot have a sensitizing dose of antibodies left in its circulating blood (Coca, 26, von Fenyvessy and Freund, 27). Furthermore, Doerr and Pick have observed that even after all demonstrable anti-

bodies have disappeared from the circulating blood in the rabbit, fatal anaphylactic shock may be produced.

Not only do we have all this evidence of the cellular site of the anaphylactic reaction, but in addition there is abundant evidence that the presence of free antibodies in the blood interferes with the anaphylactic reaction (Weil, 28) presumably by binding the antigen before it can reach the "sessile" receptors within the cells. This is the explanation of the fact that repeated injection of guinea pigs with the antigen renders them immune rather than hypersensitive, and that a single large dose of antigen sensitizes less successfully than a small dose of the same antigen.

It may safely be said that at least the essential features of acute anaphylactic shock depend upon intracellular reaction between antigen and antibodies that have been withdrawn from the circulating blood and fixed within the cells. Whether the reaction which presumably occurs between antigen and the free antibodies in the circulating blood also plays any part whatever in anaphylaxis, is still undetermined. Zinsser (1) believes that the "radical cellular interpretation of all phases of the phenomena of anaphylaxis goes too far." Several observers have reported that when suitable quantities of antigen and antibody are used, it is sometimes possible to secure passive sensitization without the usual period of incubation; indeed certain workers have stated that simultaneous injection of antigen and antiserum may give rise to immediate anaphylactic reaction, especially in rabbits. The chief objection to these experiments is that the supposed positive results are obtained only occasionally, are usually very slight in character and without the necessary regularity to be convincing evidence of a fundamental principle, and Weil doubted the true anaphylactic character of such reactions as have been observed. Friedemann obtained the best results with simultaneous intravenous injections of antigen and antiserum in the proportions which were found to give the best precipitin reactions in vitro, which suggests the possibility that the symptoms observed in the injected rabbits were not those of true anaphylaxis, but anaphylactoid phenomena produced by capillary occlusion from the precipitate, or possibly from agglutination of red cells or fibrin formation incited by the precipitate, for it has been shown by Doerr that intravenous injection of antigen-antibody precipitates produces severe symptoms.

On the other hand, there is no doubt that antigen-antibody reactions do produce, at least in vitro, substances that are eminently injurious,

especially on intravascular injection, and it seems reasonable indeed to believe that such substances may be produced in the typical anaphylaxis reaction and play some part in it, even if we grant that the typical acute anaphylactic shock depends on reaction within certain tissue cells. For example, Manwaring and Kusama (29) found that although isolated lungs from sensitized guinea pigs exhibited strong bronchial constriction when perfused with the specific antigen, they gave still stronger reactions when the blood of the sensitized animal was present in the perfusion fluid. Some authors have advanced the hypothesis that the more chronic manifestations of anaphylaxis (such as fall of temperature in guinea pigs, the splanchnic engorgement and hemorrhagic enteritis in dogs) depend on humoral antigen-antibody reaction. Weil's demonstration (25) that during the latent period immediately following injection of immune serum, despite the presence of abundant antibodies in the blood, no anaphylaxis reaction can be obtained, either acute or chronic, makes difficult of acceptance the hypothesis that humoral reactions play any important part in anaphylaxis.

"ANAPHYLATOXIN" FORMATION AND ITS RELATION TO ANAPHYLAXIS

For a time in the history of the study of the anaphylaxis reaction the cause of the intoxication clearly seemed to lie in the products of cleavage of foreign proteins into toxic elements by the enzymes of the blood or the tissues. This "anaphylatoxin" hypothesis fitted most of the known facts so well, and was so perfectly logical, that it seemed almost inevitable, yet at the present time it appears to be untenable, in the face of existing evidence, as the final explanation of anaphylaxis. It rested fundamentally upon three sets of observations. First, the demonstration by Vaughan that from almost any protein a highly poisonous substance can be produced by cleavage under suitable conditions, and that this substance produces effects in animals much resembling the phenomena of anaphylactic shock or subacute anaphylactic intoxication. Second, the similarity of the effects produced by intravenous injection of Witte's "peptone" and other products of protein cleavage, to those of anaphylaxis. Third, the demonstration by Friedemann and Friedberger of the highly poisonous character of the products of reaction in vitro of antigen, antibody and complement. Taken together these observations seemed to indicate plainly enough that anaphylaxis was a typical case of poisoning by products of specific

proteolysis of antigens by serum complement after sensitization of the antigen by the specific antibody. Friedberger especially developed this hypothesis, although others had preceded him in producing intoxication of animals by injecting the products of antigen-antibody-complement reaction.

Friedberger showed that such toxic products were formed when precipitates, resulting from the reaction of antigen and specific precipitin (or bacteria or other cells that had been sensitized with the specific cytolytic amboceptor), were digested for a suitable period with serum complement. Also that the toxic product produced symptoms resembling those of anaphylaxis when injected intravenously into animals, and that these symptoms were the same irrespective of the nature of the antigen used, which is one of the characteristic features of anaphylaxis as well as of Vaughan's products of protein cleavage. Furthermore, too long digestion, or excessive sensitization, failed to produce the "anaphylatoxin," which supported the view that the poison represented an early stage in protein cleavage, which was destroyed by further digestion.

It was soon found, however, that this so-called anaphylatoxin formation was not a specific result of antigen-amboceptor-complement reaction, as identical toxic effects are produced with normal serum that has been digested with complement in the presence of absolutely inert materials, such as kaolin, barium sulphate and agar. Jobling and Peterson found that merely extracting the lipoids from normal serum and then letting it digest itself resulted in the same type of toxicity, and they agree with Bronfenbrenner (30) in attributing all the positive results in this type of experiment to a removal or inhibition of anti-enzymes present in normal serum, so that proteolysis then sets in. Even the clotting of blood or plasma may produce changes which render it highly toxic, even for the animal from which it came, with the same anaphylaxis-like manifestations that are produced by the toxic sera in the typical anaphylatoxin experiment.

Despite the undeniable fact that blood serum may be made highly toxic when digested with complement under various conditions, and that the symptoms which follow intravascular injection of such toxic sera resemble those of anaphylactic shock in many particulars, nevertheless the responsibility of such a production of toxic products of protein cleavage for the explanation of anaphylactic shock fails of proof. Some of the points that have been advanced against this so-called humoral anaphylatoxin theory are the following:

1. It does not fit with the latent period of passive sensitization. However, intracellular formation of anaphylatoxin might account for this phenomenon.

2. Complement is not essential, since animals deprived of free complement in the circulating blood may still give anaphylactic reactions. Here again, one may suggest the presence of intracellular or reserve complement.

3. All attempts to prove that complement is a proteolytic ferment have so far failed.

4. "Anaphylatoxin" activity has been produced in serum by digestion in the absence of complement, in the absence of antigen, and in the absence of antibody. On the other hand, if antigen and the specific antibody are injected simultaneously into the opposite jugular veins of a guinea pig, the animal shows no evidence of intoxication.

5. In the anaphylatoxin experiments the existence of capillary embolism or endothelial intoxication has not been excluded, and there is reason to believe that most of the observed symptoms are anaphylactoid rather than anaphylactic.

6. All attempts to demonstrate that the blood of animals in anaphylactic shock contains a poison responsible for the observed manifestation, have failed (see Weil, 31).

The theory of the dependence of anaphylactic intoxication upon the formation of non-specific poisons from proteins by some sort of ferment reaction, received its most severe blow in the clear-cut experiments of Dale (32) with the guinea pig uterus. If we grant, as it seems we must, that stimulation of nonstriated muscle is an essential, and probably the essential feature of acute anaphylactic shock, the Schultz-Dale method portrays the reaction without the possible invalidating effects of capillary thrombosis or agglutination emboli. The fact that, as Dale points out, the specific antigen, when introduced into a bath containing the muscle strip, produces a reaction *immediately it comes in contact with the muscle*, and quite as promptly as a preformed, diffusible stimulant, such as histamine, pilocarpine or pituitary extract, is difficult to reconcile with the theory that the stimulating agent is liberated through proteolysis. Moreover, says Dale,

> Apart from the absence of a coarse latent period, the rest of the time relations of the effect are very difficult to harmonize with any form of ferment theory. On such a theory, one would expect a gradual onset and slow rise to a maximum. But the onset is sudden, and the rate of progress to the maximum, when sensitiveness is fully developed and the dosage not too minute, is apparently limited

only by the contraction rate of the plain muscle. After a few minutes at the maximum the effect begins to decline, and the muscle is then insensitive to further doses. In the form of the contraction curve, as in the brief latency, the effect recalls that of a powerful stimulant drug added directly to the bath.

These observations seem to exclude not only the humoral site of anaphylaxis reaction, but also to rule out the possibility of even an intracellular digestion of a sensitized antigen by a ferment, whether serum complement or intracellular proteolytic enzymes.

But we cannot escape the fact that the manifestations of anaphylactic shock resemble in all respects those of an acute intoxication; furthermore, that histamine, the substance which produces the picture most closely resembling that of typical anaphylaxis, is a product of protein cleavage (see Abel and Kubota, 33; Dale, 34; Hankè and Koessler, 96). Not only does histamine cause bronchial spasm in guinea pigs, obstruction to pulmonary circulation in rabbits and fall of blood pressure in dogs, but applied to the skin or mucous membranes, it causes marked local urticaria resembling closely that of skin reactions in sensitized persons, and it does all these things in extremely minute dosage, comparable with the dosage of proteins used in anaphylactic reactions. Furthermore, its antecedent amino acid, histidine, is present in every known complete protein. Other pure chemical products of protein cleavage, such as methyl guanidine, have more or less similar effects. The chief respects in which histamine fails to account for all the phenomena of anaphylaxis are:

1. It fails to desensitize sensitized animals or tissues yet produces strong reactions in the uterus strip that has been thoroughly desensitized (Dale, 32).

2. Histamine does not produce the temperature reactions usual in anaphylaxis.

3. Histamine does not produce the changes in coagulability of the blood usual in anaphylaxis.

4. Quinine augments the susceptibility of sensitized animals to the foreign protein but does not affect the intoxication produced by histamine (M. I. Smith, 35).

Nevertheless, histamine seems to have a synergetic relation to anaphylactic shock, probably because the points of attack of histamine and anaphylactic reactions are identical (35). In respect to item one of the above list, we would not expect histamine to desensitize if it is the active product of an antigen-antibody reaction, since it is this reaction alone that is prevented by desensitization. As to items three

and four, it may well be that these phenomena result from other products of the antigen-antibody reaction, since presumably many different substances are produced. The effect of quinine (item four) might be found to be an influence on the antigen-antibody reaction if this possibility were investigated.

Moreover, in the so-called Abderhalden reaction we have evidence that protein cleavage does occur in mixtures of immune serum and specific protein, and that in this process highly active toxic substances are produced (Bronfenbrenner, 30). For whatever may be said concerning the specificity of this reaction, there undoubtedly does commonly occur a greater amount of proteolysis in such mixtures with the specific antigen than if some other protein is present. A careful study of the Abderhalden reaction (dialysis method) in my laboratory by Elsesser (36), using Osborne's purified vegetable proteins, showed that in spite of many atypical, irregular and illogical results, "there is an obvious tendency for a substrate to react more often and yield stronger reactions when tested against its homologous immune serum, than when tested against a heterologous immune serum."

Also, we cannot afford to overlook the important fact that racemized proteins, which are characterized by being incapable of attack by enzymes in vitro, or of being digested and metabolized in vivo, are also incapable of serving as antigens, although derived from proteins which in the original state are highly antigenic.

It being established that immune sera do cause proteolysis of specific antigens, or at least produce diffusible cleavage products when in contact therewith, and since the intermediate products of proteolysis are unquestionably toxic, how can we discard entirely the possibility that such substances do arise, and play at least some part, in anaphylaxis, even if we can exclude proteolysis as the responsible factor in the immediate contraction of non-striated muscle both in vivo and in vitro? Furthermore, since the so-called anaphylatoxins are so readily produced, and exert such a profound physiological effect, their importance in human pathology must be considerable, even if they are not responsible for typical acute anaphylactic shock. Such observations as those of Quenu and Delbet, Cannon (37) and others, on the relation of disintegration of traumatized muscle tissue to traumatic shock, come to mind in this connection.

Although we are justified in defining sharply the limits of what shall be considered as true anaphylaxis in discussing and studying that topic, phenomena which we must exclude as outside those limits are not

therefore any less important. They merely are other problems, and presumably related problems, but how much bearing they have on specific anaphylaxis, if any, we do not know. From this open viewpoint as a contribution to our knowledge of serum toxicity, the extensive studies of Novy and his collaborators (38) are of interest. They corroborate and extend the findings of others, that the serum of a guinea pig may be made toxic for guinea pigs after incubating with agar, inulin, or small formed particles, such as trypanosomes or inorganic precipitates, so that from 1 to 3 cc. may be a lethal intravenous dose. Rat serum, which is toxic to guinea pigs even when untreated (3 to 4 cc. sometimes being fatal) may have its toxicity enhanced until 0.25 cc. is lethal. Also, agar and other substances which render serum poisonous in vitro are also very toxic when injected intravenously, as little as 9 mgm. of agar per kilo of guinea pig being fatal. But even unformed substances may render serum toxic, e.g., peptone solutions, and distilled water is said to produce "anaphylatoxin" in rat serum, which Sachs (39) would explain by the precipitation of the serum globulins by the distilled water, thus disrupting the colloidal equilibrium and permitting the formation of toxic forms of colloids. But the proof that such phenomena are the same as those underlying the specific antigen-antibody reaction of anaphylaxis has not been furnished, and the term anaphylatoxin is misleading, especially as we have as yet no proof that a toxic material is formed in and responsible for true anaphylaxis reactions. Moreover, the "anaphylatoxins" thus formed (better called "serotoxins") seem to have little effect on rabbits, although these animals are far from insusceptible to anaphylaxis. Nor can we reconcile the observation that the mere coagulation of blood renders the serum highly toxic, with the known facts of anaphylaxis. Unquestionably the readiness with which blood becomes poisonous is an important phenomenon, and presumably it plays an important part in pathology, but that this phenomenon is responsible for, or even related to, anaphylaxis, has yet to be demonstrated. Novy and DeKruif make this statement: "It will be seen that the phenomenon of anaphylatoxin production, which includes anaphylaxis, is not connected with the problem of immunity except so far as it shows the ready response of a labile blood constituent to an alien substance." But anaphylaxis *is* a definite immunological phenomenon, and if the phrase "which includes anaphylaxis" is omitted from this statement most immunologists will agree, accepting the independence of so-called anaphylatoxin formation from immunological reactions in general and from anaphylaxis in particular.

But if we discard the anaphylatoxin theory of anaphylaxis, we are left without an explanation of the very striking phenomena of anaphylactic shock, for no satisfactory substitute hypothesis has been proposed. Dale (32) suggests that "A disturbance of the conditions of colloidal solution is set up in the muscle fibre," which is not altogether satisfactory, although it may well be true. Weil speaks of a "cellular discharge" which is even more vague. Danyz (40) has proposed the hypothesis that anaphylaxis is merely an intracellular or intravascular "indigestion," because of the impossibility of transforming colloidal antigen into crystalloids, but this does not fit at all the known facts of the character of the reaction in isolated tissues. None the less, the fact that slight disturbances in the equilibrium of plasma colloids render them highly toxic is an outstanding fact, and it becomes easily understandable that a similar alteration in colloidal equilibrium within the cell protoplasm may produce equally profound intoxication of the cell, and at present there is a growing tendency to seek an explanation of anaphylaxis in the domain of colloidal chemistry (see Zinsser, 1, Sachs, 39).

PATHOLOGICAL PHYSIOLOGY

Anaphylactic reactions have been produced in many species of mammals, but only in the guinea pig, rabbit and dog has the process been thoroughly studied. The striking fact soon developed that in each species the phenomena were different, and always the same in that species without relation to what antigen was being used. No matter how widely separated in biological origin or chemical character the antigenic protein might be, the symptoms produced were always the same, a fact which gave support to the hypothesis that the poison responsible was derived from the proteins of the sensitized animal itself. Auer and Lewis demonstrated that in the guinea pig the typical respiratory difficulty was caused by a spasm of the bronchial musculature. Anatomical studies showed that the guinea pig has an exceptionally highly developed musculature in its bronchi, the finer bronchioles being practically nothing but muscular tubes, possessing also a thick folded mucous membrane. More recent experiments have shown that this spasm of the bronchioles is not accompanied by a corresponding spasm in the blood vessels, since perfusion fluids pass through the distended, anaphylactic guinea pig lung without obstruction.

In the rabbit this lung inflation is not observed, or at least not to any corresponding degree, the symptoms being chiefly circulatory.

There is a fall in arterial blood pressure, slowing of the heart beat, with marked distention of the right side of the heart. Auer believed that these changes resulted from a direct effect on the muscle of the right ventricle, and the isolated hearts of both rabbits and guinea pigs do give reactions in vitro. Coca (41) found that during anaphylactic shock the rabbit's lung develops a greatly increased resistance to the passage of perfusion fluids, with embolism and thrombosis excluded, apparently from a spasm of the muscular coat of the arterioles, which explains the observed symptomatology. Here again histological study offers an explanation, since the pulmonary arteries of the rabbit present a remarkable degree of muscular development, analogous to that of the guinea pig bronchioles.

The symptoms produced in the dog are again quite different and distinctive. Here the liver and splanchnic circulation show the most marked effects, becoming tremendously engorged with blood, so that the systemic blood pressure falls because depleted of blood. Weil (42) showed that this was a local reaction, for when he injected the antigen into one of the branches of the portal vein supplying a single liver lobe, the congestion was limited to this lobe. This hepatic effect has generally been attributed to an action on the liver cells, especially since it is accompanied by alterations in the coagulability of the blood, and other evidences of altered hepatic function, such as reduced capacity to form urea from ammonium lactate (Rumpf, 43). A more plausible and logical explanation has been advanced by Simonds (44) who states that the walls of the hepatic veins of the dog differ from those of all other animals in the great development of their musculature, inferring that the hepatic and splanchnic congestion are the result of spasm of the hepatic veins. As yet the necessary tests to show that occlusion of the hepatic veins does occur, and that this of itself can account for the observed phenomena in canine anaphylaxis, have not been reported.

If this explanation is correct, the characteristic features of acute anaphylactic shock in different species depend merely on differences in the distribution of non-striated muscle in the different species. We have abundant experimental evidence that unstriated muscle of all tissues or organs is stimulated to contraction in the anaphylactic reaction, which explains the micturition and defecation, and perhaps the erection of the hairs and the exophthalmos observed in anaphylactic shock. Whether the marked changes in the heart action, sometimes in the nature of heart block, revealed in the anaphylactic rabbit

by electrocardiographic methods (Auer and Robinson, 45) depend on spasm of the coronary arteries, on reaction of the heart muscle itself, or on the pulmonary obstruction, is not known. Presumably the stimulation of unstriped muscle tissue is universal [thus, in the dog severe bronchial constriction does occur even with the liver and other abdominal viscera excluded from the circulation (Pelz and Jackson, 46)], and the symptoms observed depend upon the degree of development of these muscles, or their strategic location, in different organs of different species.[2] Corroborating this view is the fact that fatal anaphylaxis reactions in man are practically limited to persons suffering from chronic asthma.[3] In such persons there is a profound thickening of the bronchial wall, together with great hypertrophy of the bronchial musculature, which facts are brought out strikingly in the study of the pathological anatomy of bronchial asthma in this laboratory by Huber and Koessler (51). Such asthmatic persons, having bronchi like those of the guinea pig, react in the same way as the guinea pig when injected with the specific antigen. On the other hand, it may occasionally happen that the manifestations may affect chiefly the splanchnic area, as in the dog (see case reported by Gurd and Roberts, 52), but the reason for this localization in these cases has not been sought in the light of Simond's observations.[4]

[2] The theory that leucocytic blocking of pulmonary capillaries is important (Werigo, 47) is not supported by many studies of the lungs in anaphylaxis, and is contradicted by the lack of any decrease in severity of reaction in guinea pigs with greatly reduced number of leucocytes from Thorium-X treatment (Corper, 48).

[3] A remarkable instance of fatal anaphylaxis in man has been reported by Jeanbrau (49). The patient had been given antitetanus serum, and three weeks later received a transfusion of blood from a man who had received a dose of antitetanus serum that very morning, death resulting in a few seconds. On the whole, however, man does not seem to be highly susceptible to anaphylaxis, at least as compared with the guinea pig; thus, Böttner (50) calls attention to the fact that collargol has had a widespread use by intravenous injection at intervals suitable to produce sensitization and shock, without recorded serious results, although it contains 25 per cent of protein and produces reactions readily in guinea pigs.

[4] Since examining Doctor Simond's preparations of the dog hepatic veins, I have been interested in the great muscular development of the human adrenal veins. Although I have not yet had an opportunity to make measurements of this material, I have often noticed the large amount of muscular tissue in the central veins of the adrenal, apparently greater than that in other veins of corresponding caliber in man. In view of the relation of epinephrin to anaphylactic shock this observation, if corroborated by actual measurements, may be of some significance.

There is no pathological anatomy of anaphylactic shock that cannot apparently be explained by such a mechanism as that described above. The anaphylactic poison, if there is one, would seem to be after the order of the alkaloidal poisons, since it produces immediate but transitory effects, without evident structural alterations in the tissues. Such anatomical alterations as are observed, such as hemorrhages or waxy degeneration of the voluntary muscles of respiration (53), are ascribable to the asphyxia and circulatory obstruction. Longcope (54) and Boughton (55) have observed lesions in the kidneys, hearts and livers of rabbits and guinea pigs subjected to repeated anaphylactic shock, as well as to repeated protein injections without shock, but in view of the frequency of spontaneous lesions in animals kept under similar conditions and subjected to much manipulation, it cannot be said positively that the lesions described resulted from anaphylactic shock, or from foreign proteins (see Bell and Hartzell, 56). Nevertheless, we have in the Arthus phenomenon a striking proof that local tissue injury may result from anaphylactic reactions, whether through direct cellular injury or protracted vascular occlusion, and so it would seem eminently probable that tissue injury may result elsewhere than in the skin under similar conditions.

Weil (57) has described changes in the liver of dogs dying within one hour of anaphylactic shock, consisting of a cloudy swelling and in one case disseminated necrosis, but it is possible that these changes may be the result of the intense asphyxia from which the liver suffers. It is difficult to understand how injury to the liver cells themselves could account for the engorgement, as Weil suggests, and equally difficult to accept is Dale's idea of a selective toxic action on the endothelial cells of the liver. Presumably the decreased coagulability of the blood depends on injury to the liver cells and release of anticoagulating substances,[5] for Weil found that blood containing the specific antigen loses its coagulability when perfused through the liver of a sensitized dog, and poisons which injure the liver produce a similar decrease in coagulability. Also, autolysis of the liver is said to be hastened in sensitized animals (95).

Apparently some change takes place in antigens which enter the anaphylactic liver, for Manwaring and Crowe (59) found that antigen perfused through the liver of a sensitized guinea pig lost its toxicity

[5] According to Bulger (58), however, the increase in antithrombin is not great, and he ascribes the observed changes in coagulability to variations in thromboplastin, together with a marked increase in fibrinolysis.

for sensitized guinea pig lungs, although normal livers did not have this effect, and Falls (60) observed that larger doses of antigen are required to produce shock when injected into the portal vein than when introduced into the jugular. Weil (31) has found evidence that antibody and antigen coexist within the reacting tissues for some time, the antigen being gradually destroyed, a process which may perhaps be accompanied by injury to the cells, but L. Loeb (61) could not find that thyroid or uterus from sensitized guinea pigs transplanted into other animals were modified in their vitality as tissue grafts, by injection of the specific antigen into the host.

Although we recognize the systemic anaphylactic reaction only as it affects non-striated muscle tissue, it may well be that this is merely because such tissue alone registers the effects in a graphic manner. We do not know whether any or all other cells of the sensitized animal are affected when they come in contact with the specific antigen. Still less do we know what happens within the sensitized muscle cell when the antigen and antibody meet. Dale has shown that the response is immediate, as when a potent diffusible drug acts on a cell, but whether the reaction changes the colloidal state of the cell, as has been vaguely suggested, or whether it causes a discharge of energy after the order of an electrical stimulus, or something entirely different, are matters concerning which we have not the slightest information.

Metabolism studies may show an increased toxicogenic destruction of protein (62), but the increase in amino acids, presumably resulting from proteolysis within the tissues of the reacting individual, is not large enough, if it occurs at all, to be significant. Thus, Auer and Van Slyke (63) were unable to find a demonstrable increase in free amino nitrogen in the anaphylactic guinea pig lung, nor do the livers of guinea pigs dying in anaphylactic shock contain appreciably more non-coagulable nitrogen than normal livers (64). However, in anaphylaxis in guinea pigs, as well as after peptone poisoning, there is a considerable increase in non-coagulable and urea nitrogen in the blood, as well as a slight increase in amino nitrogen, but it is not known whether this comes from the tissues or from the antigen-antibody reaction in the blood, although the former seems more probable (see Hisanobu, 65). Manwaring (66) found that no appreciable loss of the antigenic protein occurred during its perfusion through the organs of sensitized animals.

DESENSITIZATION AND ANTI-ANAPHYLAXIS

An important and characteristic feature of the anaphylactic reaction is the refractory condition which the recovered animal exhibits immediately thereafter, provided that the amount of antigen injected has been sufficiently large, and isolated muscle strips exhibit the same phenomenon. If the reaction is produced by minimal quantities of antigen, the animal may still react to a second dose of the same antigen. Evidently this condition of desensitization depends upon an exhaustion or saturation of the antibodies. The desensitization is quite as specific as the acute reaction itself, for if the guinea pig has been sensitized to two different proteins and recovers from the shock induced by one of them to which it thus becomes entirely refractory, it will still react to the second protein. I have had much occasion to take advantage of this phenomenon in determining the purity of protein preparations, and in studying the relations of such proteins, and have found it to exhibit a reliable specificity. It was noted early in this work, however, that the reaction to the second protein is seldom as severe as it would be if it were a primary reaction. This has been repeatedly confirmed by others, and Dale (32) found that the isolated uterus from an animal sensitized to more than one antigen showed a similar decrease in reactivity after a maximal reaction to one of these antigens. Presumably this is due at least in part to exhaustion of the reacting mechanism, and perhaps in part, in the case of intraperitoneal injections, to an impaired absorption because of inflammatory reactions and obstructed lymphatics.

Desensitization can be accomplished without anaphylactic shock if the antigen is introduced slowly enough, which is usually the case when the injection is subcutaneous. Conversely, the production of observable anaphylactic shock requires that the toxic dose of antigen reach the antibodies within a brief period.

Weil (67) made a quantitative study of desensitization following passive sensitization with standardized amounts of antibody, and found that the quantity of antigen needed to produce a reaction in a partially desensitized pig did not bear a constant relation to the amount of unsaturated antibody presumably left after the first reaction, as shown by the following table:

Sensitization: 0.3 cc. immune serum intraperitoneally

GUINEA PIG SERIES	DESENSITIZING DOSE OF HORSE SERUM	MINIMAL ANAPHYLACTIC DOSE OF HORSE SERUM, INTRAPERITONEALLY
	cc.	cc.
1	0.005	0.1
2	0.01	0.5
3	0.02	0.5
4	0.05	0.5

As the minimum anaphylactic dose of antigen after partial desensitization is much larger than before partial desensitization, it would seem that the reactivity or affinity of the antibody had been much reduced in the desensitizing process. This alteration in quantitative relationship between antigen and antibody is distinctly different from the fixed quantitative relations observed between precipitinogen and precipitin, and Weil pointed out the resemblance to the Danyz phenomenon observed in toxin-antitoxin neutralization; i.e., the changed quantitative relationship produced by adding antitoxin to toxin in small fractions instead of a single neutralizing quantity. As he stated,

> The same antibody, when in solution as precipitin, combines with antigen quantitatively, and in strictly constant proportions, to form precipitate; when attached to the cell, as sensitizing antibody, it combines with antigen in varying proportions. The living cell, therefore, modifies its properties.

Coca (1), however, makes the valid suggestion that these observations can be explained by the assumption that the sensitizing serum contains antibodies of varying avidity for antigen, whereby those with the greatest avidity are saturated with the first partial desensitizing dose, leaving the less reactive antibodies to unite with the second dose of antitoxin. These suggestive and important quantitative studies have not been extended since the untimely death of Doctor Weil removed one of the most brilliant and productive students of anaphylaxis.

Sensitized animals may be refractory to anaphylactic reactions not only because of saturation or exhaustion of the fixed antibodies, but because the presence of free antibodies in the circulating blood prevents the antigen from coming in contact with the intracellular antibodies, i.e., the condition of anti-anaphylaxis. There has been much confusion in the literature because of the careless use of the term "anti-anaphylaxis" to cover all refractory conditions. This term should logically be applied only to a resistance due to antibodies, using the

term desensitization for the condition which it describes, as indicated in previous paragraphs.

It is this form of antibody resistance, true specific anti-anaphylaxis, which accounts for the failure to obtain anaphylactic shock in guinea pigs immunized to a foreign protein by repeated injections. This assumption, which was made early in anaphylaxis work, seems to have been converted into a certainty by the demonstration of Manwaring and Kusama (29) that the isolated organs of such immunized guinea pigs, washed free of circulating antibodies, are very highly sensitized to the specific antigen. Weil (68) further showed that if small doses of antigen are injected subcutaneously into passively sensitized guinea pigs that have been given intravenous injections of large protective doses of antiserum, the uterus is not desensitized as it otherwise would be, since the antigens are kept from uniting with the intracellular antibodies.

Antisensitization is a somewhat similar phenomenon described by Weil (69). If a guinea pig is given a single dose of rabbit serum several days before a sensitizing dose of serum from a rabbit immune to a foreign protein, the usual passive sensitization does not take place. This is explained by the development in the guinea pig of antibodies to the rabbit serum, which protect the guinea pig's tissues from the antibodies of the immune rabbit serum. In proof of this conclusion is the fact that such preliminary injection with rabbit serum does not prevent passive sensitization with the serum of a guinea pig immunized to foreign protein. Also, Manwaring and Kusama (29) showed that in immunized guinea pigs the bronchial musculature is highly sensitized but that the presence of the blood of the immune animal will prevent reactions with the specific antigen in lung perfusion experiments.

Another form of refractoriness, which is essentially a desensitization, may occur when a large sensitizing dose is given. Differences may be observed even when the larger sensitizing doses are not very large; thus Thomsen (70) states that a sensitizing dose of 0.004 cc. of serum produces a maximum sensitization more quickly than 0.1 cc., although the final maximum degree of sensitization is the same with each dose. This seems explainable on the basis of the persistence of antigens in the blood during the time of formation of the antibodies and their fixation within the cells, whereby they are saturated with the antigen gradually as fixation takes place, without at any one time sufficient reaction taking place to be observable. Weil believed that antigen

and antibody might coexist in the cells without union, as they are known to do in the circulating blood. Of course, when a very large sensitizing dose of antigen is given there may be engendered sufficient antibodies to produce the condition of antianaphylaxis from the excess antibodies in the blood.

Still a different form of interference with sensitization has been described by Julian Lewis (71), who found that an amount of protein that will produce a marked anaphylactic sensitization when injected alone into a guinea pig, will fail to do so if injected together with, or twenty-four hours after, a much larger amount of another protein. Thus, in a series of guinea pigs sensitized with 0.1 cc. horse serum mixed with decreasing quantities of dog serum from 2 cc. to 0.001 cc., no reactions were obtained in the animals receiving from 1 to 2 cc. of horse serum, severe reactions but with recovery in those receiving from 0.25 to 0.01 cc. dog serum, as compared with promptly fatal reactions in the animals that received no dog serum.

The serum from a rabbit immune to horse serum, which will markedly sensitize a guinea pig to horse serum, will fail to do so if injected with, or twenty-four hours after, a large dose of another protein.

These results may be explained by the conception that the number of receptors in the body that can unite with a foreign protein is limited. The inhibiting protein, if present in large amount, combines with all, or almost all, of these receptors. Hence, another protein injected with it, or after it, is prevented from being combined in sufficient amount to stimulate the active production of antibodies. And when a large amount of protein is injected with or after a sensitizing dose of immune serum, the combination of the latter with the cell receptors, which is necessary for passive sensitization, is prevented in the same way.

I have found that the condition of antisensitization may be produced through feeding (72) as well as by injection, and presumably this condition explains a number of phenomena observed both in experimental animals and in man.

Guinea pigs bred from mothers fed with oats were, as soon as weaned, put upon a diet of egg albumen and carrots. Other young pigs from the same stock were raised upon oats and carrots. The latter animals, after reaching a weight of 250 to 300 grams, did not give anaphylactic reactions when injected with 0.05 gram of a protein obtained from raw oats, and if given small doses, such as ordinarily given for sensitizing, they were not rendered sensitive to subsequent injections of 0.05 gram of oat protein. Some of the pigs which were raised to 200 to 250 grams weight without oats were found to give a typical reaction of moderate severity when injected once with 0.05 gram oat protein, apparently from passive sensi-

tization conferred by the mother. Others gave no reaction. After the animals fed without oats were somewhat older, 350 to 400 grams, they reacted much less strongly or not at all to oat protein, as if this inherited passive sensitization were passing off, as passive sensitization normally does; such pigs, if given sensitizing doses of oat protein, are found to be sensitive to this protein three weeks later, giving well-defined reactions of moderate severity. Hence the conclusion seems warranted that if guinea pigs are raised on oat proteins they cannot be made to give anaphylactic reactions with oat proteins, but if raised without oats they may be sensitized to oat proteins, just as they can be to other proteins not usually in their food. These experiments support experience obtained previously with zein, that guinea pigs become immune to the chief vegetable proteins of their food.

Guinea pigs raised from the time of weaning on a diet of egg protein (Merck's dried egg albumen) and carrots were found to give strong anaphylaxis reactions when injected with egg albumen between the thirtieth and sixtieth days, but later they reacted less strongly, and after the one hundredth day of feeding they gave but slight reactions to 0.1 gram dried egg albumen. At this time sensitization with egg albumen can be obtained to only a slight degree, such guinea pigs given injections of egg albumen showing but slight reaction to a subsequent dose of egg albumen, while control pigs fed on oats and carrots gave severe, usually fatal, reactions to corresponding injections of egg albumen. Apparently, then, daily absorption of animal protein in the food at first renders guinea pigs hypersensitive to this protein, but if the feeding is kept up for a long enough period the animals become refractory to the food protein and are so immunized that they cannot be sensitized to this protein.

A series of guinea pigs which were raised on bread and cow milk for ten weeks were found at the end of this time to be still highly sensitive to milk, dying promptly when given 1 to 3 cc. of milk intraperitoneally. Apparently this length of feeding is not sufficient to render guinea pigs immune to milk. These results are not at all in harmony with those of Besredka (73), who did not succeed in sensitizing with milk by either oral or rectal administration, but did find that sensitized guinea pigs were made refractory to intracerebral injections of milk if previously given milk by either of these routes. They are corroborated, however, by the observation of Schlutz and Larson (74) that the blood of infants exhibiting the exudative diathesis when injected into guinea pigs renders them passively sensitive to milk.

Non-specific transitory reduction of the intensity of anaphylactic reactions may be produced by intravenous injection of numerous substances shortly before giving the intoxicating dose. Peptones, trypsin, various inorganic salts, urine and foreign proteins other than those used in sensitizing, are among these non-specific desensitizers, and their mechanism of action is not understood. Kopaczevski and Vahram (75) have found that sodium oleate has this effect to a marked degree, which they ascribe to a lowering of surface tension of the blood, on the hypothesis that anaphylactic shock depends upon a plugging of

capillaries by colloidal flocculation. Although perfusion experiments demonstrate that such gross capillary occlusion does not occur in the anaphylactic lung, yet the various items of evidence that changes in the colloidal state of the blood are of importance in the phenomena of "anaphylatoxin" intoxication make this observation of significance. The protective action of NaCl has usually been ascribed to its interference with complement action, but Richet (76) attributes it to its action on nerve cells, and others would ascribe it to the effect on the colloidal state of the cells and fluids. Thomsen (70) refers non-specific interference with the anaphylaxis reaction to a reduction in the speed of reaction between antigen and antibody, whereby a greater amount of antigen is found necessary to produce shock, but no explanation is offered as to why the speed of reaction is reduced.

THE SPECIFICITY OF THE ANAPHYLAXIS REACTION

If we limit the evidence on this subject, as we properly should, to that obtained in unquestionable typical anaphylaxis with protein antigens, it may be said that the anaphylaxis reaction exhibits exactly the same degree of specificity that we obtain with the complement fixation or precipitin reactions. In all cases group reactions are obtained when sera or extracts of tissues, etc., derived from closely related species, are used, and likewise, as emphasized by Magnus and others (20) and confirmed for the isolated uterus (by Dale, 32), increasing the antigenic doses tends to broaden the scope of the reaction.

Experiments with such hopelessly complex mixtures as are furnished by serum, organ extracts, or even egg whites and milk, cannot be expected to yield much valuable information concerning specificity. A common error in the literature is the statement that the anaphylaxis reaction, or some other immunological reaction, is not absolutely specific, since slighter reactions are obtained with sera of related species, etc. What is not specific in such experiments is the antigen used, not the reaction. If the antigens are isolated proteins, purified as completely as possible, striking illustrations of specificity may be demonstrated both by sensitizing and by desensitizing with specific proteins. For such work the proteins from seeds and nuts seem to be especially useful, for commonly they represent stored proteins, apparently set apart with a small number of other proteins as compared with the conditions in blood and tissues; furthermore, many of them are crystallizable, some are soluble in alcohol, and others present strik-

ing solubility characteristics, so that they are especially susceptible of separation and purification. Of the animal proteins those that resist heat coagulation, such as casein and ovomucoid, or those that crystallize, of which egg albumin is the chief example (for the crystallizable hemoglobins are not antigenic) are the best materials.

Working with such proteins, it has been possible to demonstrate conclusively that biological origin is not, *per se*, the thing that determines the immunological specificity of a given protein, but its chemical composition. The fact that the sera or tissues of different species can commonly be distinguished from one another by immunological reactions, indicates therefore that as a rule the proteins of different species are chemically different from the proteins of other species, even when these proteins have similar functions and exhibit close physical and chemical similarities, e.g., horse serum and ox serum. But there are many exceptions to this rule of immunological differences between the proteins of different species, e.g., goat casein and cow casein show to a considerable degree interchangeable immunological reactions, whereas they show a more distinct specificity against the serum proteins of the same species as furnished the casein. Thus, cow milk casein can be differentiated even from lactalbumin of the same species more sharply than from casein of goat's milk by complement fixation (Bauer), and cow casein and goat casein sensitize mutually to one another (72). A study of the proteins of hens' eggs by the anaphylaxis reaction showed that five distinguishable antigens could be differentiated from one another, and, furthermore, that these corresponded to five different proteins that had been differentiated by chemical methods, namely, ovomucoid, ovovitellin, albumin, and two different globulins (72).

Extended studies with milk proteins have also developed the same fundamental fact, that antigens which can be distinguished from one another by means of the anaphylaxis reaction are usually found to be readily distinguishable from one another by chemical means (77). The fact that different protein fractions of serum show stronger reactions when the same fraction is used as both sensitizing and intoxicating doses than when different fractions are used (Dale and Hartley, 78; Kato, 79) probably indicates a similar relationship.

The study of vegetable proteins also brings this out clearly, and furthermore indicates that we may have chemically distinct although similar proteins, which show corresponding distinctions and relations. Thus, hordein of barley is chemically similar to gliadin of wheat and rye, although showing sufficient chemical differences to establish it as a

distinct protein, but gliadin of wheat is chemically indistinguishable from gliadin of rye. Guinea pigs sensitized with gliadin from wheat or rye give strong anaphylactic reactions when injected with hordein of barley, but not as strong as with the homologous proteins, and the same results are obtained with injections in the reverse order. After such cross reactions, however, the recovered animals are not found to be desensitized to the homologous proteins, which indicates either that there are two or more individual proteins in the preparations of hordein and gliadin, one of which is common to both, or else the presence in gliadin and hordein of common and specific reactive groups. The chemical evidence is in favor of the latter conclusion. On the other hand, the chemically indistinguishable gliadins of rye and wheat interact against one another as if they were one and the same protein (7). Other examples of crossed reactions between unlike proteins have been observed; for example, legumin from pea renders guinea pigs as sensitive to legumin from vetch as it does to itself, and vice versa, and legumin from other seeds shows close immunological relation. On the other hand, a chemically distinguishable protein from another leguminous plant, glycinin from soy bean, does not sensitize to vetch legumin, or vice versa (80). The so-called natural proteoses of seeds show many striking illustrations of the same sort (11).

These and other experiments have led to the conclusion that

> the entire protein molecule is not necessarily involved in the specific character of the anaphylaxis reaction, but this is developed by certain groups contained therein, and that one and the same protein molecule may contain two or more such groups. It may well be that the intact protein molecule is involved in the reaction (for there is but little evidence that anything less than an intact protein molecule is capable of producing the typical reaction), but that certain groups determine the specificity (Wells and Osborne, 81).

Also, "since chemically similar proteins from seeds of different genera react anaphylactically with one another, while chemically dissimilar proteins from the same seed in many cases fail to do so, we must conclude that the specificity of the anaphylaxis reaction depends upon the chemical structure of the protein molecule" (80). Corroborative evidence has been furnished with precipitin reactions by Landsteiner and Lampl (82).

As to what groupings in the protein molecule determine the specificity, little is known. Obermayer and Pick (83) believed that the specificity characteristic of species was determined by the aromatic radicals, since iodizing or diazoting proteins removed this specificity

when tested by the precipitin reaction. Iodized proteins tested by the anaphylaxis reaction, however, I have not found to have altered in range of specificity from the original proteins (8).

That the anaphylaxis reaction is a less delicate means of determining specificity than the precipitin reaction, and especially the complement fixation reaction, is shown by the fact that when animals are immunized to hordein a serum of low antibody titer reacts only with hordein, but with antihordein serum of high titer both precipitin and complement fixation reactions are obtained with gliadin of wheat or of rye and passive sensitization is given with the same proteins (20).

Probably the more general use of the uterus strip method will enhance the delicacy of anaphylaxis work in the future, since this offers less uncontrollable factors than experiments with the intact animal. A good illustration of this is furnished by a recent report from Dakin and Dale (84). Previous workers have stated that the egg proteins of different species of birds are not readily distinguishable by anaphylaxis reactions, or at least that they show a less marked specificity than serum (for review, see 72). Welsh and Chapman found by the precipitin reaction, using the saturation test, that antiserum for any egg white contains a general avian antisubstance reacting with all sorts of bird egg proteins, and an independent antisubstance specific for the species. By the anaphylaxis reaction, however, such tests as have been made have shown a striking absence of specificity. Dale and Hartley found, in preliminary experiments, that crystallized albumins from hen eggs and from duck eggs sensitized mutually to one another even with the guinea pig uterus method, but by using quantitative methods and by checking up with the desensitizing test, it was later possible for Dale to detect differences between the two. The importance of this observation lies especially in the studies carried out by Dakin on the chemistry of these two albumins. When hydrolyzed, their amino acid content is very similar, but when the proteins are first racemized and then hydrolyzed it is found that there are distinct differences in the degree of racemization of the leucine, aspartic acid and histidine. There being reasons for believing that the amino acids which escape racemization occupy the terminal positions in the peptid chains of which the protein molecule is built, this result indicates that there are at least structural or spatial differences in these two proteins. In other words, a close relationship by immunological tests is here associated with chemical similarity, and a slight difference in chemical structure is found which presumably accounts for a slight

immunological difference that can be detected only by the most sensitive methods. This point of view is quite in harmony with that developed by Osborne and myself from our studies of the immunological specificity of the vegetable proteins. Although not based on the anaphylaxis reaction, but on precipitins, the corroborative results of Landsteiner and Lampe (82) may be recalled here; namely, that if one derivative (halogenized, azotized, etc.) of a protein reacts with another sort of derivative, the position in the molecule of the substituted radicals is identical or closely related. That is to say, cross reactions depend on chemical relationships, and the specificity is determined by relatively small portions of the large antigen molecule.

The observation that the location in the molecule of definite groups is indicated by their immunological reactions can best be explained as depending on spatial correspondence of antigen and antibody, just as Emil Fischer assumed for the specific action of ferments in his comparison to "lock and key." Here again we get evidence that both chemical composition and spatial relations are concerned in determining specificity. Presumably there are also innumerable isomeres that cannot be distinguished by our present methods, which correspond to the racial and individual differences which are so obvious and yet not to be detected by serum reactions.

Contemplating the possible number of variations in the arrangement of the amino-acids in a protein which the great number of these radicals provides, there is no difficulty in understanding the existence of an almost limitless number of specific distinctions between proteins. Abderhalden, indeed, calculated that the 20 amino acids we find in proteins could form at least 2,432,902,008,176,640,000 different compounds, and this without including possible compounds varying in quantitative relations. A contribution to the chemical basis of specificity has been made by Kossel (85), who finds certain relations in the proportions and groupings of the scanty number of amino-acids that make up the protamines and histones of sperm, to be characteristic of the sperm of certain species and families.

THE RELATION OF ANAPHYLAXIS TO SERUM DISEASE, TO DRUG HYPERSENSITIVENESS AND TO HAY FEVER

At present the evidence in respect to these topics is so contradictory as to defy positive conclusions. In respect to certain acute serum reactions, such as one in which a person with "horse asthma" dies in a few moments of bronchial spasm after receiving an injection of horse

serum, it seems evading the obvious to attempt to interpret the occurrence in any other way than as true anaphylactic shock, resulting from a specific antigen-antibody reaction; and as such a sensitized person usually exhibits also typical acute local reactions immediately after intracutaneous introduction of the most minute amounts of horse serum, or of other horse proteins as well, it seems difficult to deny, at least in such a case, that the cutaneous reaction of hypersensitiveness represents again a true specific antigen-antibody reaction, and is a true manifestation of anaphylaxis. Furthermore, it is sometimes possible to desensitize a person to the protein to which he is sensitive, removing both the systemic and cutaneous reactivity.

A particularly interesting demonstration by means of passive sensitization from man to man, of the true antigen-antibody nature of horse asthma and the specific skin reaction accompanying it, has been described by Ramirez (86). A man who had never had asthma, hay fever, urticaria, or any similar condition, received a transfusion of 600 cc. of blood from a man with typical horse asthma, who gave a cutaneous reaction to horse dandruff in 1–50,000 dilution. Two weeks later the transfused patient went for a carriage ride and within five minutes had a typical attack of asthma, which recurred when he again went into the park, and a skin test gave a positive reaction to horse dandruff diluted 1–20,000 but not to any of numerous other proteins. Also we have the passive transfer of sensitivity to milk when the blood of infants with exudative diathesis is injected into guinea pigs (Schlutz and Larson, 74).

But as soon as we leave such cases as these, and consider ordinary serum sickness with its cutaneous manifestations and other symptoms which follow at an uncertain period of time after a serum injection, often when this is the first injection of such a serum, the resemblance to anaphylaxis is much less distinct. Coca seems to have little hesitation in eliminating serum sickness entirely from the category of anaphylaxis reactions, laying particular emphasis upon the many points serum disease has in common with drug hypersensitiveness, which he believes cannot possibly be true anaphylaxis. He says "serum allergy exhibits no peculiarity that can serve as a criterion of separation of this condition from that of drug allergy and there is, thus, no occasion offered by the manifestation of serum allergy to seek an underlying mechanism that would be different from that operative in drug allergy, that is, one dependent on an antibody-antigen reaction." Hence he eliminates from the domain of anaphylaxis all the conditions manifested by cutane-

ous hypersensitivity, including the tuberculin reaction, hay fever, food and drug idiosyncracies, etc.

Kolmer (87), on the other hand, in his review on the cutaneous reactions, states that there are true anaphylactic skin reactions, "due to the interaction of a specific anaphylactic antibody and specific anaphylactogen largely within or without the cells, and with the formation of diffusible irritant capable of producing acute hyperemia, edema and leucocytic infiltration of the skin." He thus accepts the theory of local anaphylaxis depending on the formation of a poison by cellular reactions to foreign proteins introduced into the skin.

The relationship of the cutaneous manifestations of serum disease to anaphylaxis is especially well supported by the studies of C. W. Wells (88) on the precipitin content of the blood of patients who have received diphtheria antitoxin, for he found that in several persons who developed such manifestations the precipitin titer fell to rise again when the rash faded, as if the precipitin had been bound in the skin and thus caused a local anaphylactic reaction. Also, Weil (31) observed in human serum sickness a fall in blood pressure and a decrease in the coagulability of the blood (89), thus adding to the resemblance of this condition to true anaphylactic reactions.

However, among other facts that seem to separate cutaneous hypersensitiveness from systemic anaphylaxis, are the results described by Krause (90) in his studies on *The Nature of Resistance to Tuberculosis.* He states that whereas, as shown by Baldwin, it is easily possible to sensitize guinea pigs to tuberculo-protein so that they will give typical systemic anaphylactic reactions, such sensitized animals do not show cutaneous hypersensitiveness to tuberculo-protein, nor are they protected against infection with the tubercle bacillus. Skin sensitiveness can only be obtained by producing an actual tuberculous infection somewhere in the animal and when this tissue reactivity has developed it is found that the animal has the power to produce acute inflammatory reactions about injected tubercle bacilli, whereby its resistance is enhanced. Fleischner, Meyer and Shaw (91) have corroborated this and find that it is equally true for typhoid bacilli and B. abortus. Guinea pigs may be highly immunized with the dead bacilli or extracts therefrom, so that their blood has a high agglutinin content, or anaphylactic sensitization may be produced, and yet no cutaneous sensitivity can be shown; but guinea pigs infected with living bacteria give positive skin tests.

In support of the view that ordinary serum sickness following injection of foreign sera is not a true anaphylactic reaction, but is rather the result of a primary serum toxicity, may be cited the observation of Kraus (92) that bovine serum seldom causes serum sickness in man as compared with its frequency after horse serum. However, it is possible that man, using beef protein as a usual article of diet, may develop an actual immunity to beef proteins, as I found guinea pigs to do with the proteins forming the chief elements of their diet.

As to hay fever, Coca, who at one time described it as "a clinical expression of a local hypersensitiveness," the view most generally held, has been compelled by the evidence more recently developed, to take the opposite view and is now convinced that hay fever is not the result of a protein sensitization. He is satisfied to accept its hereditary character as an adequate explanation, although this does not in any way clear up the pathogenesis of the condition. The many observations of successful desensitization by repeated injection of pollen proteins is an outstanding fact which surely establishes a relation to anaphylactic sensitization, as also the close relationship to the many forms of asthma that are accompanied by skin hypersensitivity to the same proteins that are responsible for the asthma, and in which desensitization may be obtained by injection of the specific exciting protein. On the other hand, desensitization is accomplished by no means so readily or so surely as in typical anaphylaxis, and even marked systemic reactions produced by relatively large desensitizing doses of pollen may fail to desensitize (Rackemann, 93). An additional point, of which much is made in discussing the relation of hay fever to true anaphylaxis, is that it is usually impossible to confer passive hypersensitivity for pollen to guinea pigs by injecting them with the serum of patients with hay fever, but this negative fact seems of little significance if we consider that even the serum of guinea pigs highly sensitized to a foreign protein usually contains too few free antibodies to confer passive sensitization to other guinea pigs. The cells of a man may well be highly sensitized to a foreign protein without there being free antibodies in his blood to produce passive sensitization.

A new feature of local reactions has recently been brought to light by Auer (97), which may throw light on some of the manifestations of cutaneous hypersensitivity. Observing that operation wounds made in sensitized dogs at the time of reinjection showed a marked inflammatory reaction with a brawny edema, the suggestion was followed up, and the following facts were observed. If the ear of a normal rabbit

is rubbed gently with xylol there occurs a moderate inflammatory reaction which disappears in a short time without serious injury to the skin. The effects are quite the same in a rabbit that has been sensitized to a foreign protein, but in rabbits that have been sensitized and then reinjected with the same protein the xylol commonly produces a violent reaction, with exfoliative dermatitis followed by dry gangrene of the tips of the ears. The explanation of this striking effect seems to be simple. The slight inflammation produced by the xylol leads to a certain amount of inflammatory exudate. In the sensitized animals which have been recently re-injected with the sensitizing protein the blood contains more or less free antigen, which is poured out into the tissues with the exudate. Here it produces a local reaction with the sensitized tissues quite the same as if it had been locally injected, resulting in the usual local reaction seen in sensitized animals. Presumably similar effects could occur in any other tissue or organ. The importance of this observation lies in the recognition of a hitherto unappreciated mechanism by which anaphylactic reactions may be caused under certain conditions, by substances which themselves do not produce anaphylaxis. Conceivably, sensitized persons at times have free antigen in their blood, especially in cases of food sensitization, and if this is so, injury to any tissue or organ might possibly determine local anaphylactic reactions in these places.

BIBLIOGRAPHY

(1) von Pirquet, C.: Arch. Int. Med., 1911, vii, 259.
Hektoen, L.: Journ. Amer. Med. Assoc., 1912, lviii, 1081.
Besredka, A.: Anaphylaxis and antianaphylaxis and their experimental foundations; English ed., by S. R. Gloyne, 1919, St. Louis.
Doerr, R.: Kolle und Wassermann's Handb., 1913, ii, 947.
Zinsser, H.: Arch. Int. Med., 1915, xvi, 223.
Coca, A. F.: Tice's Practice of medicine, Prior & Co., New York, 1920, 107.
Boughton, T. H.: Journ. Lab. Clin. Med., 1920, v, 597.
Wells, H. G.: Chemical pathology, 4th ed., Philadelphia, 1920, 191.
(2) Karsner, H. T., and Hanzlik, P. J.: Journ. Pharm. Exper. Therap., 1919, xiv, 229, 379, 425, 449, 463, 479.
(3) Gay, F. P., and Robertson, T. B.: Journ. Exper. Med., 1913, xviii, 535.
Schmidt, C. L. A.: Univ. Cal. Publ. Pathol., 1916, ii, 157.
(4) Wells, H. G.: Zeitschr. f. Immunität., 1913, xix, 599. ("Betanucleoproteins" seem to have a thermostable antigenic capacity; see Journ. Biol. Chem., 1916, xxviii, 11.)
(5) Schmidt, C. L. A., and Bennett, C. B.: Journ. Infec. Dis., 1919, xxv, 207.
(6) Starin, W. A.: Journ. Infec. Dis., 1918, xxiii, 139; corroborated by Kahn and McNeil: Journ. Immunol., 1918, iii, 277.

(7) Wells, H. G., and Osborne, T. B.: Journ. Infec. Dis., 1911, viii, 66.
(8) Wells, H. G.: Journ. Infec. Dis., 1908, v, 449; Journ. Amer. Med. Assoc., 1908, l, 527.
(9) Wells, H. G.: Journ. Infec. Dis., 1909, vi, 536; 1911, ix, 147.
(10) Wells, H. G., and Osborne, T. B.: Journ. Infec. Dis., 1915, xvii, 259.
(11) Dochez, A. R., and Avery, O. T.: Journ. Exper. Med., 1917, xxvi, 477.
(12) Elliott, C. H.: Journ. Infec. Dis., 1914, xv, 501.
(13) Schmidt, C. L. A.: Journ. Immunol., 1920, v, 258.
(14) Ten Broeck, C.: Journ. Biol. Chem., 1914, xvii, 369.
(15) Landsteiner, K.: Biochem. Zeitschr., 1919, xciii, 106.
(16) Fink, E. B.: Journ. Infec. Dis., 1919, xxv, 97.
(17) Abderhalden, E.: Zeitschr. physiol. Chem., 1912, lxxxi, 315.
(18) Zunz, E.: Biochem. Journ., 1916, x, 169; Journ. physiol. et path. gén., 1917, xvii, 449; Arch. internat. de physiol., 1919, xv, 179, 192.
(19) White, B.: Journ. Med. Res., 1914, xxx, 393.
(20) Lake, G. C., Osborne, T. B., and Wells, H. G.: Journ. Infec. Dis., 1914, xiv, 364.
(21) Weil, R.: Journ. Immunol., 1916, i, 19.
(22) Coca, A. F., and Kosakai, M.: Journ. Immunol., 1920, v, 297.
(23) Zinsser, H.: Journ. Exper. Med., 1913, xviii, 219.
(24) Larson, W. P., and Bell, E. T.: Journ. Infec. Dis., 1919, xxiv, 185; see also Coca, A. F.: Journ. Immunol., 1919, iv, 269.
(25) Weil, R.: Journ. Med. Res., 1913, xxx, 87.
(26) Coca, A. F.: Zeitschr. f. Immunität., 1914, xx, 622.
(27) von Fenyvessy, B., and Freund, J.: Zeitschr. f. Immunität., 1914, xxii, 59.
(28) Weil, R.: Journ. Med. Res., 1913, xxx, 299.
(29) Manwaring, W. H., and Kusama, Y.: Journ. Immunol., 1917, ii, 157.
(30) Bronfenbrenner, J.: Journ. Exper. Med., 1915, xxi, 480.
(31) Weil, R.: Journ. Immunol., 1917, ii, 399.
(32) Dale, H. H.: Journ. Pharm. Exper. Therap., 1913, iv, 167.
(33) Abel, J. J., and Kubota, S.: Journ. Pharm. Exper. Therap., 1919, xiii, 243.
(34) Dale, H. H.: Johns Hopkins Hosp. Bull., 1920, xxxi, 257, 310.
(35) Smith, M. I.: Journ. Immunol., 1920, v, 239.
(36) Elsesser, O. J.: Journ. Infec. Dis., 1916, xix, 655.
(37) Cannon, W. B.: Compt. Rend. Soc. Biol., 1918, lxxxi, 850.
Delbet: Rev. de Chir., 1919, xxxviii, 309.
(38) Novy, F. G., and De Kruif, P. H.: Journ. Amer. Med. Assoc., 1917, lxviii, 1524; this being a summary of ten articles in the Journ. Infec. Dis., 1917, xx, 499 to 854.
(39) Sachs, H.: Kolloid Zeitschr., 1919, xxiv, 113.
(40) Danyz, J.: Journ. Infec. Dis., 1918, xx, 427.
(41) Coca, A. F.: Journ. Immunol., 1919, iv, 219.
(42) Weil, R.: Journ. Immunol., 1917, ii, 429.
(43) Rumpf, F.: Zeitschr. Immunität., 1918, xxvii, 489.
(44) Simonds, J. P.: Journ. Amer. Med. Assoc., 1919, lxxiii, 1437.
(45) Auer, J., and Robinson, G. C.: Journ. Exper. Med., 1913, xviii, 450.
(46) Pelz, M. D., and Jackson, D. E.: Journ. Lab. Clin. Med., 1918, iii, 387.
(47) Werigo, B.: Compt. Rend. Soc. Biol., 1916, lxxiv, 87.

(48) Corper, H. J.: Journ. Infec. Dis., 1919, xxv, 248.
(49) Jeanbrau: quoted by Giraud: Arch. Méd. et Pharm. militaire, 1918, lxxix, 369.
(50) Böttner, A.: Deutsch. Arch. klin. Med., 1918, cxxv, 1.
(51) Huber, H. L., and Koessler, K. K.: Arch. Int. Med., 1921.
(52) Gurd, F. B., and Emrys-Roberts, E.: Lancet, 1920, excviii, 762.
(53) Wells, H. G.: Centralbl. allg. Pathol., 1912, xxiii, 945.
(54) Longcope, W. T.: Journ. Exper. Med., 1913, xviii, 678; 1915, xxii, 793.
(55) Boughton, T. H.: Journ. Immunol., 1919, iv, 213.
(56) Bell, E. T., and Hartzell, T. B.: Journ. Infec. Dis., 1919, xxiv, 618.
(57) Weil, R.: Journ. Immunol., 1917, ii, 525.
(58) Bulger, H. A.: Journ. Infec. Dis., 1918, xxiii, 522.
(59) Manwaring, W. H., and Crowe, H. E.: Journ. Immunol., 1917, ii, 517.
(60) Falls, F. H.: Journ. Infec. Dis., 1918, xxii, 83.
(61) Loeb, L.: Journ. Immunol., 1917, ii, 557.
(62) Major, R. H.: Deutsch. Arch. klin. Med., 1914, cxvi, 248.
(63) Auer, J., and Van Slyke, D. D.: Journ. Exper. Med., 1913, xviii, 210.
(64) Barger, G., and Dale, H. H.: Biochem. Journ., 1914, viii, 670.
(65) Hisanobu, K.: Amer. Journ. Physiol., 1920, l, 357.
(66) Manwaring, W. H., Kusama, Y., and Crowe, H. S.: Journ. Immunol., 1917, ii, 511.
(67) Weil, R.: Journ. Immunol., 1917, ii, 469.
(68) Weil, R.: Journ. Med. Res., 1913, xxx, 299.
(69) Weil, R.: Zeitschr. Immunität., 1913, xx, 199.
(70) Thomsen, O.: Zeitschr. Immunität., 1917, xxvi, 213.
(71) Lewis, J. H.: Journ. Infec. Dis., 1915, xvii, 241.
(72) Wells, H. G.: Journ. Infec. Dis., 1911, ix, 147.
(73) Besredka, A.: Ann. Inst. Pasteur, 1909, xxiii, 166.
(74) Schlutz, F. W., and Larson, W. P.: Arch. Pediatrics, 1918, xxxv, 705.
(75) Kopaczevski, W., and Vahram, A.: Compt. Rend. Acad. Sci., 1919, clxix, 250.
(76) Richet, C., Brodin, P., and St. Girons, F.: Compt. Rend. Acad. Sci., 1919, clxix, 9.
(77) Osborne, T. B., and Wakeman, A. J.: Journ. Biol. Chem., 1918, xxxiii, 7 and 243.
(78) Dale, H. H., and Hartley, P.: Biochem. Journ., 1916, x, 408.
(79) Kato, Y.: Mitt. Med. Fak. Univ. Tokio, 1917, xviii, 195.
(80) Wells, H. G., and Osborne, T. B.: Journ. Infec. Dis., 1916, xix, 183.
(81) Wells, H. G., and Osborne, T. B.: Journ. Infec. Dis., 1913, xii, 341.
(82) Landsteiner, K., and Lampl, H.: Biochem. Zeitschr., 1918, lxxxvi, 343.
(83) Obermayer, Fr., and Pick, E. P.: Wien. klin. Wochenschr., 1906, xix, 327.
(84) Dakin, H. D., and Dale, H. H.: Biochem. Journ., 1919, xiii, 248.
(85) Kossel, A.: Zeitschr. physiol. Chem., 1913, lxxxviii, 163.
(86) Ramirez, M. A.: Journ. Amer. Med. Assoc., 1919, lxxiii, 984.
(87) Kolmer, J. A.: Johns Hopkins Hosp. Bull., 1917, xxviii, 163.
(88) Wells, C. W.: Journ. Infec. Dis., 1915, xvi, 63.
(89) Shattuck, H. F.: Arch. Int. Med., 1917, xx, 167.
(90) Krause, A. K.: Amer. Rev. Tubere., 1917, i, 65.

(91) Kleischner, E. C., Meyer, K. F., and Shaw, E. B.: Amer. Journ. Dis. Chil., 1919, xviii, 577.
(92) Kraus, R.: Rev. del Inst. Bact. Buenos Aires, 1919, ii, 1.
(93) Rackemann, F. M.: Boston Med. Surg. Journ., 1920, clxxxii, 295.
(94) Kritchewsky, J. L.: Journ. Infec. Dis., 1918, xxii, 101.
(95) Haskinoto, M., and Pick, E. P.: Arch. exper. Path. u. Pharm., 1914, lxxvi, 89.
Fenyvessy, B., and Freund, J.: Biochem. Zeitschr., 1919, xcvi, 223.
(96) Hanke, M. T., and Koessler, K. K.: Journ. Biol. Chem., 1920, xliii, 521–579.
(97) Auer, J.: Journ. Exper. Med., 1920, xxxii, 427.

Anaphylaxis Reactions with Purified Proteins from Milk

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ANAPHYLAXIS REACTIONS WITH PURIFIED PROTEINS FROM MILK*

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In the course of a study of the proteins of cow's milk by one of us (O),[1] four proteins were isolated in a high degree of purity, and their individuality established by means of the anaphylactic reaction. Evidence of the essentially chemical basis of immunologic specificity, as emphasized in our previous communications on the immunologic reactions of the vegetable proteins,[2] thus received further support. Our experiments were performed chiefly to determine the chemical individuality of the various protein preparations under investigation, and the completeness of their separation from one another. However, since these results had an important bearing on problems of immunologic specificity, numerous additional experiments have been made from this standpoint with these carefully isolated proteins.

As one of the most readily available protein-containing solutions, milk perhaps has been used in immunologic experiments with greater frequency than any other material except blood and egg white. The ease with which casein can be separated from milk in a relatively pure condition and its ready solubility in dilute alkali have made this protein particularly suitable for use when a pure isolated protein was desired. Hence many references are to be found in the literature dealing with the immunologic reactions of casein. It is evident, however, from the published accounts, that the casein used in much of this work was not so carefully purified as it should have been. Furthermore, in only a few instances in which the proteins of the whey [3] have been used, have these been separated from each other with the care necessary to secure sufficiently pure preparations. As we have found no complete review of the literature of the immunologic reactions of the several proteins of milk, we here present a brief synopsis in approximately chronologic order.

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[1] Osborne and Wakeman: Jour. Biol. Chem., 1918, 33, pp. 7 and 243.

[2] Wells and Osborne: Jour. Infect. Dis., 1913, 12, p. 341; 1916, 19, p. 183.

[3] The term whey is here used to designate the solution of the milk solids from which casein has been removed by precipitation with acid.

IMMUNOLOGIC EXPERIMENTS WITH MILK AND MILK PROTEINS

Although the earliest experimental work on immunologic reactions with protein solutions dates to 1897, apparently isolated casein was not used until 1901. Moro,[4] Hamburger,[5] and Levene[6] independently, and almost simultaneously, published observations on the precipitin reactions with casein. Moro reported that by immunizing with pure casein, from either human or cow's milk, he obtained a "specific lactoserum," but he did not describe its properties further. He also reported that precipitins for cow's milk gave reactions with goat's milk, but not with human milk, which was not in agreement with the earlier work on milk precipitins by Wassermann and Schütze.[7] The important observation was made that heating milk one-half hour at 100 degrees did not destroy its reactivity or its antigenic property.

Hamburger[5] found that "lactalbumin" (which included all the proteins in solution after removing the casein) could be distinguished from casein by the precipitin reaction. Antilactalbumin serum (or, more properly, antiwhey serum) gave reactions with whole milk and with lactalbumin, but not with casein, and anticasein serum reacted with whole milk and casein, but not with whey. He prepared his casein by precipitating with acetic acid and then washing the precipitate free from "albumin." He also used Schlossmann's method of filtering off the casein through a "Thonzellenfilter," and stated that the "albumin" in the filtrate behaved somewhat differently from that obtained by precipitating out the casein.

In connection with other work, Levene[6] merely reported that immune serum for milk gave precipitates with milk, casein, "milk albumin" and beef serum.

Fuld[8] stated that whey obtained by filtering milk through porcelain filters did not react with antimilk serum. Since this is not in harmony with observations by others, it probably means that his immunization was sufficient to produce precipitins only for casein, and not for the other less abundant milk proteins. His statement that heated milk or casein solution did not produce precipitins has been shown to be incorrect. His antiserum for cow's milk did not react with goat's milk or with isolated casein from human milk.

Müller,[9] in a study of the relation of specific antiserum precipitins to rennin, observed that antimilk serum did not precipitate casein except in the presence of calcium salts, although it united with the casein in their absence. He found no evidence that the precipitation of casein by antiserum was accompanied by the splitting off of proteins similar to those of whey. In another article[10] he stated that the products of digestion of casein with pepsin or trypsin did not produce precipitins either for themselves or for casein, although paracasein and iodocasein immunization gave precipitins that precipitated casein. Lactoserum did not precipitate paracasein.

Gengou[11] isolated casein, lactalbumin and lactoglobulin from cow's milk, finding that the casein and globulin gave complement fixation and precipitin reactions with antimilk serum, but the albumin did not react. The antiserum for cow's milk reacted with goat's and sheep's milk, and less strongly with human and horse's milk.

[4] Wien. klin. Wchnschr., 1901, 14, p. 1073.
[5] Ibid., 1901, 14, p. 1202.
[6] Med. News, 1901, 79, p. 981.
[7] Verein f. inn. Med., Verinsbeilage, July 26, 1900, p. 178; Schütze. Ztschr. Hyg. u Infektionskr., 1901, 36, p. 5.
[8] Hofmeister's Beitr., 1902, 2, p. 425.
[9] Arch. f. Hyg., 1902, 44, p. 126.
[10] Centralbl. f. Bakteriol., I. O., 1902, 32, p. 521.
[11] Ann. de Inst. Pasteur, 1902, 16, p. 734.

Schlossmann and Moro,[12] who discussed the methods used in the separation of milk proteins, recognized that the so-called milk "albumin" is a mixture of globulin and albumin, containing no phosphorus, which statement later studies have shown to be incorrect as far as globulin is concerned. They found also that the casein and albumin fractions each gave specific reactions for the species from which they were derived, and also that globulin from human serum reacted with antihuman-milk serum, but not with anticow's-milk serum. Antiserum for human milk gave precipitates with human serum.

Amberg,[13] who reprecipitated his preparations, found that the precipitin reaction with milk or casein did not depend on the presence of either inorganic or casein salts of calcium, thus contradicting Müller. He observed no differences between the reaction with anticasein and antimilk serums when used against casein, milk, or lactalbumin.

Fleischer[14] immunized animals with purified casein from animals of various species (rabbit, cat, dog, horse, goat, cow and human), and found that the antiserums gave precipitin reactions with both the homologous and foreign caseins, although with quantitative differences in favor of the homologous caseins. Antiserum for cow's casein gave about two-thirds as much precipitate with goat's casein as with cow's casein, and a good precipitate was obtained with horse's casein, but only a trace with dog's, cat's and rabbit's casein, and none with human casein. Antiserum for human casein gave only relatively slight reactions with all the other caseins.

In their early studies of anaphylaxis, Rosenau and Anderson[15] made some experiments with milk, obtaining no reactions when cow's milk was injected into guinea-pigs that had been sensitized with either human or dog's milk, but positive results were given when the sensitization had been caused by sheep's milk. The first examination of isolated casein by means of the anaphylaxis reaction is reported by Besredka,[16] who stated that casein gave the same results as milk. The whey was found to sensitize to milk sometimes, but it did not always give reactions in animals sensitized to milk. Perhaps the difficulty here lies in the use of too small amounts of the whey (1/20 to 1/4 c c), which has a very small protein content.

Wells[17] observed that purified casein was even more active in anaphylactic reactions than whole milk, for 0.1 to 0.25 gm. of purified cow's casein usually produced fatal reactions when injected into the peritoneum of sensitized guinea-pigs, while doses of 5 to 10 c c of whole milk containing 0.15 to 0.30 gm. of casein were seldom followed by fatal reactions. The activity of casein solutions was not materially impaired by heating at 100 degrees for 25 minutes. Animals sensitized to goat's milk casein reacted severely when given cow's casein, or conversely, which agrees with Bordet's results with whole milk, and Fleischer's precipitin reactions with goat's and cow's casein.

A related observation on the lack of species specificity with casein is furnished by Michaelis and Rona,[18] who injected cow's casein subcutaneously into dogs and guinea-pigs that had recently been actively secreting milk, and observed a notable swelling of the mammary glands, without marked constitutional symptoms. Some reaction was obtained in virgin animals. This they interpret as the production of a heightened secretory activity which induced

[12] München. med. Wchnschr., 1903, 50, p. 597.
[13] Jour. Med. Research, 1904, 12, p. 341.
[14] Roussky Wratsch., 1908, 7, p. 1638.
[15] Jour. Med. Research., 1907, 16, p. 391.
[16] Ann. Inst. Pasteur, 1909, 23, p. 166.
[17] Jour. Infect. Dis., 1908, 5, p. 480; 1911, 9, p. 147.
[18] Pflüger's Arch., 1909, 121, p. 163.

the mammary glands to excrete the injected casein, although in none of the animals did actual secretion of milk follow the injection. On the other hand, Felländer[18a] was unable to sensitize guinea-pigs to guinea-pig's milk or to extracts of their mammary glands.

Bachrach[19] failed to secure a good differentiation between casein, whey and beef serum by anaphylactic reactions. As he used the same sized doses for both sensitizing and intoxicating, his observations are not valuable.

The use of the complement fixation reaction with casein was introduced by Kollmeyer,[20] who obtained distinct differentiation of casein from whey by this method.

Graetz[21] studied particularly the relation of milk to colostrum, and the only feature of his work of interest in this connection is the evidence obtained that colostrum whey contains proteins which seem to be identical with those of the serum. Other workers have obtained evidence of similar significance, indicating the whey proteins to be closely related to the serum proteins. Thus, Bauer[22] concluded that casein can be differentiated by complement fixation from the protein of the whey; that casein of different species of animals gives common group reactions, as also do the whey proteins, but that only the casein can be differentiated from the serum proteins of the same species. Bauereisen[23] finds that the complement-fixation method, like the precipitin reaction, does not permit an absolute differentiation of milk proteins from other proteins of the same individual, but that both methods show casein to be much less closely related to the serum proteins than are serum proteins to whey proteins. He also recognized the probability that the failure to distinguish sharply between the isolated proteins depends on defects in the method of their preparation.

The quantitative differentiation of milk proteins demonstrable by complement fixation is great enough to be recognized by the less sensitive anaphylaxis reactions, as indicated by the results obtained by Kleinschmidt.[24] He separated casein, globulin and albumin from cow's milk, but in his description of the process used does not indicate that he reprecipitated his preparations. His "casein" was everything that came down with sufficient acetic acid; the "globulin" was the precipitate produced by saturating with magnesium sulfate after removing the casein with NaCl, and the "albumin" was the precipitate obtained by adding acetic acid to the filtrate from the globulin. He found that anaphylaxis reactions (intracardiac) gave distinct differentiation between these three proteins. Casein sensitized to itself as well as whole milk, but did not sensitize to beef serum, lactalbumin or lactoglobulin. Experiments in the reverse order were almost equally specific. There were some slight crossed reactions, especially between globulin and casein, and a distinct protection (anti-anaphylaxis) reaction in all cases, probably due to incomplete separation of the proteins used. Guinea-pigs sensitized with cow's casein did not react with human casein; no other foreign caseins were tried. Animals sensitized with either cow's lactalbumin or lactoglobulin reacted with bovine serum, and conversely; those sensitized with lactalbumin reacted with lactoglobulin, and conversely. These last results do not warrant the statement made

[18a] Ref. in Ztschr. f. Immunität., Ref., 1912, 6, p. 851.
[19] Vierteljahr. gericht. Med., 1910, 40, p. 235.
[20] Ztschr. f. Biol., 1910, 54, p. 64.
[21] Ztschr. f. Immunität., 1911, 9, p. 677.
[22] Berl. klin. Wchnschr., 1910, 47, p. 830.
[23] Ztschr. f. Immunität., 1911, 10, p. 306.
[24] Monatschr. f. Kinderheilk., 1911, 10, p. 402.

in the author's summary: "In anaphylaxis experiments the proteins of cow's milk, casein, albumin and globulin, can be differentiated from one another." There are quantitative differences in his reactions, to be sure, but those are not constant. It is quite evident that the author was working with imperfectly purified protein preparations. A similar difficulty is found in the work of Heuner[25] who used commercial (Merck) cow's milk albumin and casein, as well as a milk albumin and a globulin prepared by a chemist for him, the description not indicating that his preparations were thoroughly purified. Nevertheless, he also reached the conclusion that globulin and albumin of milk are distinct from casein, and that colostrum and mastitis milk are more nearly related to the blood serum antigens than is normal milk. The differentiation was less marked in anaphylaxis experiments than with complement fixation.

Bauer and St. Engel[26] separated casein, globulin and albumin in the same way as Kleinschmidt, but reprecipitated the globulin three times, although the albumin seems not to have been reprecipitated. Using the complement-fixation reaction, they found distinct quantitative differences between all three proteins. Thus, with an anticasein (bovine) serum, complement fixation was complete, or nearly so, in the following dilutions of 0.5% solutions: casein, 1:256; globulin, 1:32; albumin, 1:16; milk, 1:64. With antiglobulin serum the figures were: casein: 1:8; albumin, 1:32; globulin, 1:16,384. With antialbumin serum the figures were: casein, 1:1; globulin, 1:8; albumin, 1:64. Similar results were obtained with the globulin or casein of human milk. The proteins of one species were not tested against the antiserums for the other species. Cow's milk globulin could not be differentiated from beef serum globulin, hence these authors believed the globulin and albumin of milk or colostrum or serum to be identical.

Klein[27] made a study of the nature of casein antiserum by the precipitin test, finding evidence that there are in such an antiserum two precipitins. One acts only in the presence of $CaCl_2$, while the other acts best in the absence of calcium. Hence the presence of two different antigen groups is postulated in the casein molecule. So far as we can learn, this important work has not been reinvestigated. The suggestion of the existence of two antigenic groups in a purified protein is, however, in harmony with the observations of Wells and Osborne[28] with purified alcohol-soluble proteins from wheat, rye and barley.

In agreement with the results of Bauer and St. Engel, it was found by Kudicke and Sachs[29] that under suitable conditions antimilk serum will, by virtue of the whey proteins, give complement-fixation reactions with blood serum of the same species, but if milk is boiled, the antiserum it engenders will react with milk but not with blood serum. (Evidently this depends on the coagulability of the whey and serum proteins and the incoagulability of casein.) Antiserum for bovine serum will give slight reactions with cow's milk boiled for not over ten minutes. Uhlenhuth and Haendel[30] had previously found that boiled milk gave anaphylactic reactions only in animals sensitized with milk, whereas raw milk caused reactions in animals sensitized with either milk or serum of the same species.

[25] Arch. f. Kinderheilk., 1911, 56, p. 358.
[26] Biochem. Ztschr., 1911, 31, p. 46.
[27] Folia microbiol., 1912, 1, p. 101.
[28] Jour. Infect. Dis., 1913, 12, p. 541.
[29] Ztschr. f. Immunit., 1914, 20, p. 316.
[30] Ibid., 1910, 4, p. 761.

Versell[31] separated casein by adding acetic acid just short of precipitation, and then saturating the milk with CO_2; the precipitate was washed five times by centrifugation. The whey proteins were not separated from one another. Using the complement-fixation reaction, he found that antiserum for cow's milk reacted with goat's milk, but less strongly than with cow's milk, and more strongly than with human milk; the difference between the caseins of the three species was much less, and most between the wheys. Human milk antiserum gave reactions with human milk diluted to 1 : 31,250, with cow's milk at 1 : 1,250, with goat's milk at 1 : 50. Antiserums for milk or whey gave slight reactions with the serum of the same animal, but anticasein serum did not, as observed by Bauer and St. Engel, and Kudicke and Sachs; also heated casein was still antigenic but heated whey was not. Whey proteins showed the same degree of species specificity as blood serum, but casein showed much less species specificity, being rather in the class of the organ-specific proteins. He found the thermo-resistance of casein is only relative, for it is inactivated if heated sufficiently long or high.

Gay and Robertson[32] found that isolated casein, as well as the "paranuclein" derived from it by peptic digestion, were both distinctly antigenic, one sensitizing against the other. Immunization with casein produced much more active precipitating and complement-fixing antiserums than did paranuclein, and these antiserums also reacted much more strongly with casein than with paranuclein. That is, partial peptic digestion reduced but did not destroy the antigenic power of casein. Completely digested casein was not antigenic. When united with protamine, it retained its antigenic power, the resulting antiserum reacting with either casein or protamine caseinate.[33] These authors believed that the paranuclein synthesized by the "reverse action" of pepsin on the products of peptic digestion of casein is an effective antigen, sensitizing and immunizing (complement fixation) both to itself and the paranuclein produced by partial peptic digestion, and thought that they had thus established the identity of the materials and also that synthesis of a true antigenic protein had been accomplished.[34]

The statement that casein is not an active antigen, made by Dale and Hartley,[35] is supported by the fact that injection of milk itself has been used extensively in nonspecific foreign protein therapy, ordinarily without harmful results, and Müller[36] states that in an extensive experience he has never observed serious symptoms. However, the next article in the same journal, by Lubiner,[37] describes a nearly fatal reaction following intramuscular injection of 10 c c of milk into a girl, 8 days after the last of 3 daily injections. Oppenheim[38] also observed a similar case of severe reaction from milk. Of some interest may be the observation that the serum of parturient and lactating women produces a stronger positive Abderhalden reaction with casein than does the serum of men or normal or pregnant women (Kastan[39]). Also we may mention that milk has hemolytic properties, as first shown by Bertarelli.

[31] Ibid., 1915, 24, p. 267.
[32] Jour. Exper. Med., 1912, 16, p. 470.
[33] Gay and Robertson: Ibid., 1912, 16, p. 479.
[34] Ibid., Jour. Biol. Chem., 1912, 12, p. 233.
[35] Biochem. Jour., 1916, 10, p. 431.
[36] Deutsch. med. Wchnschr., 1918, 44, p. 545.
[37] Ibid., p. 547.
[38] Wien. klin. Wchnschr., 1917, 30, p. 1519.
[39] München. med. Wchnschr., 1914, 61, p. 2126.

Pfaundler and Moro found that it contains hemolytic complement. Bauer, Kopf and Sassenhagen observed that this is especially abundant in colostrum, which was corroborated by Schmidt (lit.).[40]

Taken all together, the results obtained by the several observers quoted seem to point definitely to the following conclusions:

1. Biologically, casein is quite as distinct from the whey proteins and the serum proteins as it is chemically.

2. The biologic reactions and chemical composition of caseins from different species of animals show close relationships.

3. The whey proteins as a whole are biologically similar to the soluble serum proteins of the animals from which they are derived. Colostrum contains a larger proportion of proteins of this type.

4. Casein from the milk of an animal of any given species shows a closer biologic relationship to the casein of another species than it does to either the whey proteins or to the serum proteins of its own species; the same is true of the chemical relations.

It is evident, however, that the amount of work done with proteins reasonably well isolated is as yet small, and in need of amplification and control.

THE CHEMISTRY OF MILK PROTEINS

In recent years the proteins of milk have received more careful study than previously, and several new facts must receive consideration in biologic work. Applying D. D. Van Slyke's amino nitrogen method, Crowther and Raistrick[41] found that from the chemical standpoint casein, lactoglobulin and lactalbumin were sharply differentiated and distinct proteins, and that they have the same composition whether prepared from colostrum or from milk. Only 0.03% of true globulin was found in milk; it was much more abundant in colostrum. As far as could be determined, the proteins designated as eulactoglobulin and pseudolactoglobulin are chemically identical. Lactoglobulin showed an amino-acid make-up similar to that of globulin from ox-blood, but marked differences were found between lactalbumin and serum albumin. These observations correspond with the results of the immunologic demonstration that casein is distinct from the whey proteins, and that the latter possess features common to the serum proteins of the same species. A series of milkings made twice daily after calving were analyzed, the average composition of the milk from 7 cows showing a steady drop in content of protein between the first and eighth milkings, the percentage of nitrogen present in the milk being shown in table 1. These figures indicate the relatively great abundance of globulin in colostrum, and its rapid reduction within the first 48 hours of lactation.

While caseins from different species do not show demonstrable quantitative or qualitative chemical differences by ordinary methods, a study of the products of racemization by Dakin's method yielded to Dudley and Woodman[42]

[40] Arch. f. Kinderheilk., 1911, 56, p. 312.
[41] Biochem. Jour., 1916, 10, p. 438.
[42] Ibid., 1915, 9, p. 97.

evidence of some structural differences. Caseins from sheep's and cow's milk consist of identical amino-acids, apparently in identical proportions, but when racemized the sheep's casein showed all the tyrosine and most of the lysine unracemized, while both were completely racemized in cow's casein.

Dale and Hartley[35] state that caseins from different species show no clear disparity of antigenic properties, but it is possible that finer quantitative methods will disclose distinct discrepancies, as was found to be the case with the albumins from hen's eggs and duck's eggs. These same authors state that the antigenic properties of casein are relatively feeble.

Osborne and Wakeman[43] found that the protein called lactoglobulin differed much in its solubilities from other globulins, and that it contained 0.24% of phosphorus, in which respect it resembled ovovitellin. In addition they obtained a small quantity of material that seemed to be a proteose, although not enough was obtained to permit positive identification. Of particular interest is the discovery that a protein is present in small amount in milk, which is readily soluble in 50-70% alcohol. This alcohol-soluble milk protein in its content of amino-acids is distinctly different in composition from casein.

We, therefore, have chemical evidence that milk contains at least 4 chemically distinguishable proteins: (1) casein, characterized by a

TABLE 1

Percentage of Nitrogen in Milk

	Total	Casein	Albumin	Globulin	Nonprotein
1st milking	2.40	0.75	0.14	1.32	0.19
4th milking	2.40	0.51	0.11	0.31	0.04
8th milking	0.65	0.46	0.05	0.12	0.02

high content of phosphorus: (2) lactalbumin, a water-soluble protein which contains no phosphorus; (3) lactoglobulin, which contains 0.24% of phosphorus; and (4) the alcohol-soluble protein recently described by Osborne and Wakeman.

EXPERIMENTAL PART

Before describing our results we would first call attention to the fact that anaphylaxis experiments should be performed only with animals that have been raised on a known dietary which does not contain the proteins under investigation, and the same should be true of the mothers, since a certain amount of active immunization may result from the food proteins, which can be passively transferred to the fetus. It has been shown by one of us[44] that young guinea-pigs fed on a given protein (egg white or milk) after a short time become sensitized to that protein so that they will give typical reactions when it is injected

[43] Jour. Biol. Chem., 1918, 33, p. 7.

[44] Wells, H. G.: Jour. Infect. Dis., 1911, 9, p. 147.

parenterally. If the feeding is long continued the animals become so immunized after a time that even a sensitizing injection of this protein will not render them hypersensitive, and a second injection has no effect. Guinea-pigs raised largely on oats, therefore, do not give good

TABLE 2

CASEIN

	Sensitizing Dose, Gm.	Intoxicating Dose, Gm.	Results	Remarks
	Casein	Casein		
1	0.001	0.05	Died, 5 minutes	
2	0.001	0.05	Died, 15 minutes	
3	0.050	0.05	Severe	Note large sensitizing dose
4	0.050	0.05	Severe	
5	0.005	0.001	Slight, doubtful	
6	0.005	0.005	Slight	
7	0.005	0.010	Died, 60 minutes	
8	0.005	0.020	Severe	Nearly died
9	0.005	0.000,1	Died, 2 minutes	Intracardiac
10	0.005	0.000,02	Moderate	Intracardiac
11	0.005	0.000,01	Doubtful	Intracardiac
12	0.005	0.000,01	Slight, moderate	Intracardiac
13	0.001	0.100	0 ?	
14	0.001	0.100	Moderate	
15	0.000,5	0.100	Severe	Nearly died
16	0.000,5	0.100	0	
17	0.000,1	0.100	Slight	
18	0.000,1	0.100	0	
19	0.000,05	0.100	0	
	Casein	Lactalbumin		
20	0.050	0.100	Slight	
21	0.005	0.050	Slight	
22	0.005	0.050	0	
	Lactalbumin	Casein		
23	0.005	0.050	0	
24	0.005	0.050	0	
	Casein	Lactoglobulin		
25	0.005	0.050	0	
26	0.005	0.050	Doubtful	
	Lactoglobulin	Casein		
27	0.005	0.050	0	
28	0.005	0.050	Slight	
	Casein	Alcohol soluble protein		
29	0.001	0.050	Slight	
30	0.001	0.050	0	
31	0.005	0.050	0	
32	0.005	0.050	Moderate	
	Alcohol soluble protein	Casein		
33	0.005	0.050	Moderate	No protection
34	0.005	0.050	Severe	No protection
35	0.005	0.050	0	No protection
36	0.005	0.050	0	No protection
37	0.005	0.050	Died, 10 minutes	
38	0.005	0.050	Slight	
	Casein	Beef serum		
39	0.005	1.0 c c	0	
40	0.005	1.0 c c	0	
	Beef serum	Casein		
41	0.1 c c	0.050	0	
42	0.1 c c	0.050	0	

reactions with oat proteins, although readily sensitized to other, unrelated vegetable proteins; and guinea-pigs raised on corn were found not to serve for reactions with proteins from corn, although readily sensitized to oat proteins. Probably this fact accounts for numerous

failures and irrational results obtained in anaphylaxis work, and must be taken into account, particularly in experiments with proteins such as those of milk, that might be present in the guinea-pig dietary. In these experiments all our guinea-pigs have been raised exclusively on a vegetable diet, as have their mothers.

In determining specificity it is also imperative that every precaution be taken to avoid contamination, especially of the sensitizing doses, with even the smallest possible quantity of the protein that is to be used for the intoxicating dose. As numerous articles discussing anaphylactic specificity do not mention these precautions, leaving the reader uncertain as to the significance of the results described, we state explicitly that in this, as in all our work, such contaminations were rigidly excluded. All glassware, after thorough washing, is kept for at least 24 hours in concentrated sulphuric acid and potassium bichromate; the needles are most thoroughly cleaned and kept in absolute alcohol.

During the course of this work several preparations of various fractions, obtained in the isolation and purification of the milk proteins, were tested by means of the anaphylaxis reaction. The following tables give the results obtained with purified preparations of the 4 known proteins. The terms used are as defined in our previous work.[45] Unless otherwise specified the injections were made by the intraperitoneal route. The interval between sensitizing and intoxicating injections was usually about from 18 to 20 days. The references to protection indicate the protection of a sensitized animal against reinjection with the homologous antigen after previous injection of some heterologous antigen.

The first nineteen experiments recorded in this table deal with the antigenic properties of casein. They show that, on the whole, casein is not so active as some other soluble proteins in producing anaphylactic sensitization and shock, but, on the other hand, its activity is by no means low. By intracardiac injection the minimum lethal dose is apparently between 0.000,02 and 0.00001 gram, but by intraperitoneal injection it requires nearer 0.010 gram to cause death, and 0.020 gram is not always fatal. Presumably this great difference in the efficiency of intracardiac and intraperitoneal injections of casein depends on the readiness with which casein is thrown out of solution, for with the vegetable proteins we have found that their intraperitoneal toxicity for sensitized animals seems to vary directly with their solubility in the body fluids.[46] As compared with its intoxicating power the sensitizing

[45] Wells, H. G., and Osborne, T. B.: Jour. Infect Dis, 1911, 8, p. 88.
[46] Wells, H. G., and Osborne, T. B.: Jour. Infect Dis., 1914, 14, p. 377.

capacity of casein seems to be very low. Table 3 offers a comparison of the anaphylactogenic activity of the milk proteins with that of other proteins as determined by us.

Experiments 20 to 24 show how completely distinct these preparations of lactalbumin and casein are from one another. The same is shown for casein and lactoglobulin by experiments 25 to 28. Experiments 29 to 38 indicate that the alcohol soluble protein is quite distinct from casein, but that our alcohol soluble protein preparation probably contains a trace of casein, since with 5 mg. sensitizing doses 2 of 4 animals gave severe reactions with casein. Since the alcohol soluble protein is obtained from casein by washing the precipitated casein with alcohol, this admixture is to be expected.

TABLE 3

COMPARATIVE ANAPHYLACTOGENIC ACTIVITY

Protein	Minimum Fatal Sensitizing Dose, Gm.	Minimum Sensitizing Dose, Gm.	Minimal Fatal Intoxicating Dose, Intraperitoneal, Gm.	Minimal Fatal Intoxicating Dose, Intracardiac, Gm.
1. Casein	0.001	0.0001	0.010	0.0001
2. Lactalbumin	0.001	0.001	0.005	0.0001
3. Lactoglobulin	0.001	0.000,01	0.010	
4. Alcohol soluble protein	0.001	0.0001	0.010	0.0002
5. Egg albumin	0.000,001	0.000,000,1	0.0005	0.000,05
6. Egg globulin	0.000,001	.....	0.0008	
7. Edestin	0.0005	0.000,000,1	0.100	
8. Squash seed globulin	0.000,000,5	0.000,000,5	0.010	
9. Brazil nut proteose	.....	.....	0.0005	
10. Soy bean proteose	.....	.....	0.002	

Experiments 39 to 42 indicate the absence in beef serum of appreciable amounts of any protein resembling casein. Elsewhere we have reported observations showing that casein will sensitize to milk, and conversely, as has been observed by others.

The first thirteen experiments of this table indicate that lactalbumin is an active anaphylactogenic substance, thus differing from the albumin fraction of horse serum, which according to numerous observers, is very defective in anaphylactogenic properties.[47] It is much less actively anaphylactogenic than egg albumin, but corresponds closely to casein.

Experiments 14 to 25 indicate that lactalbumin and lactoglobulin are entirely distinct proteins, but that our preparations are not completely separated, for while 5 mg. sensitizing doses give little or no crossed reactions, 50 mg. sensitizing doses of either preparation will

[47] Dale and Hartley: Biochem. Jour., 1916, 10, p. 408. See Kato, Mitt. med. Fak. Univ. Tokio, 1918, 18, p. 195.

do so. As the globulin and albumin separations merely depend on the fact that the globulin comes down in a neutral saturated solution of magnesium sulphate, while the albumin is precipitated from the filtrate of this precipitation by acidification, it is remarkable to find that the separation can be made so nearly complete. The fact that

TABLE 4

Lactalbumin

	Sensitizing Dose, Gm.	Intoxicating Dose. Gm.	Results	Remarks
	Lactalbumin	Lactalbumin		
1	0.005	0.070	Died, 17 minutes	
2	0.005	0.070	Died, 20 minutes	
3	0.100	0.050	Severe	Note large sensitization dose
4	0.050	0.050	Died, 30 minutes	
5	0.001	0.050	Died, 20 minutes	
6	0.000,1	0.050	0	
7	0.000,01	0.050	0	
8	0.005	0.030	Died, 35 minutes	
9	0.005	0.020	Moderate	
10	0.005	0.010	Died, 70 minutes	
11	0.005	0.005	Died, 50 minutes	
12	0.005	0.000.1	Died, 20 minutes	Intracardiac
13	0.005	0.000.05	0	Intracardiac
	Lactalbumin	Lactoglobulin		
14	0.005	0.070	0	
15	0.005	0.070	0	No protection
16	0.005	0.050	0	
17	0.005	0.050	0	
18	0.100	0.050	Severe	Note large sensitization dose
19	0.050	0.050	Severe	
	Lactoglobulin	Lactalbumin		
20	0.005	0.070	Moderate	Partial protection
21	0.005	0.070	Slight	Partial protection
22	0.050	0.050	Severe	Note large sensitization dose
23	0.050	0.050	Severe	
24	0.005	0.050	0	No protection
25	0.005	0.050	0	Partial protection
	Lactalbumin	Casein		
26	0.005	0.050	0	
27	0.005	0.050	0	
	Casein	Lactalbumin		
28	0.005	0.050	Slight	
29	0.005	0.050	0	
30	0.050	0.100	Slight	Note large sensitization dose
	Lactalbumin	Alcohol soluble protein		
31	0.005	0.050	0	
32	0.005	0.050	0	
	Alcohol soluble protein	Lactalbumin		
33	0.005	0.050	0	
34	0.005	0.050	0	
	Lactalbumin	Beef serum		
35	0.005	0.5 c c	0	No protection
36	0.005	0.5 c c	Slight	
	Beef serum	Lactalbumin		
37	0.1 c c	0.070	0	Partial protection
38	0.5 c c	0.050	0	No protection

we are here dealing with distinctly different proteins is certainly demonstrated. Experiments 26 to 30 show that lactalbumin is a distinct protein, and that it can be separated from casein almost perfectly.

The complete distinction of lactalbumin from the alcohol soluble protein is established by experiments 31 to 34, while the distinction from casein has already been commented on.

That the albumin fraction of cow's milk is distinct from the protein called albumin, or any other protein, of cow serum, is shown by experiments 35 to 38. This agrees with the demonstration by Crowther

TABLE 5

LACTOGLOBULIN

	Sensitizing Dose, Gm.	Intoxicating Dose. Gm.	Results	Remarks
	Lactoglobulin	Lactoglobulin		
1	0.005	0.070	Died, 24 minutes	
2	0.005	0.070	Died, 10 minutes	
3	0.005	0.050	Died, 25 minutes	
4	0.005	0.020	Slight	
5	0.005	0.015	Severe	Nearly died
6	0.005	0.010	Severe	Died after 8 hours
7	0.005	0.010	Moderate	
8	0.005	0.005	Slight	
9	0.050	0.050	Moderate	Note large sensitization dose
10	0.050	0.050	Severe	
11	0.005	0.000,1	0	Intracardiac
12	0.005	0.000,2	Doubtful	Intracardiac
13	0.005	0.000,5	Slight	Intracardiac
14	0.001	0.050	Died, 40 minutes	
15	0.000,1	0.050	Severe	
16	0.000,01	0.050	Severe	
17	0.000,001	0.050	Slight	
	Lactoglobulin	Lactalbumin		
18	0.005	0.070	Slight	
19	0.005	0.070	Moderate	
20	0.050	0.050	Severe	Note large sensitization dose
21	0.050	0.050	Severe	
22	0.005	0.050	0	Not protected
23	0.005	0.050	0	Not protected
	Lactalbumin	Lactoglobulin		
24	0.005	0.050	0	Not protected
25	0.005	0.050	0	Not protected
26	0.100	0.050	Severe	Note large sensitization dose
27	0.050	0.050	Severe	
28	0.005	0.050	0	
29	0.005	0.050	0	
	Lactoglobulin	Casein		
30	0.005	0.050	0	Not protected
31	0.005	0.050	Slight	Not protected
	Casein	Lactoglobulin		
32	0.005	0.050	0	
33	0.005	0.050	Doubtful	
	Lactoglobulin	Alcohol soluble protein		
34	0.005	0.050	0	
35	0.005	0.050	0	
	Alcohol soluble protein	Lactoglobulin		
36	0.005	0.050	0	
37	0.005	0.050	0	
	Lactoglobulin	Beef serum, c c		
38	0.005	0.5	Severe	
39	0.005	0.5	Died, 25 minutes	
40	0.005	1.0	Died, 32 minutes	
41	0.005	1.0	Died, 42 minutes	
42	0.001	0.5	Died, 35 minutes	
43	0.001	0.5	Died, 20 minutes	
	Beef serum, c c	Lactoglobulin		
44	1.0	0.060	Slight	
45	1.0	0.040	Moderate	
46	0.5	0.050	Severe	
47	0.5	0.050	Moderate	
48	0.1	0.050	Died, 25 minutes	
49	0.1	0.050	Severe	

and Raistrick [41] of the chemical distinction between bovine lactalbumin and bovine serum albumin.

TABLE 6

ALCOHOL SOLUBLE PROTEIN

	Sensitizing Dose, Gm.	Intoxicating Dose, Gm.	Results	Remarks
	Alcohol soluble protein	Alcohol soluble protein		
1	0.005	0.050	Died, 10 minutes	
2	0.005	0.050	Died, 15 minutes	
3	0.005	0.050	Slight	
4	0.005	0.050	Died, 5 minutes	
5	0.005	0.020	Slight	
6	0.005	0.015	Died, 25 minutes	
7	0.005	0.010	Died, 25 minutes	
8	0.005	0.005	Severe	
9	0.005	0.000,1	Moderate	Intracardiac
10	0.005	0.000,2	Died, 2 minutes	Intracardiac
11	0.005	0.000,2	Died, 4 minutes	Intracardiac
12	0.001	0.100	Died, 40 minutes	
13	0.000,1	0.100	Severe	
14	0.000,01	0.100	0	
15	0.000,001	0.100	0	
	Alcohol soluble protein	Casein		
16	0.005	0.050	Moderate	
17	0.005	0.050	Severe	
18	0.001	0.050	0	
19	0.001	0.050	0	
20	0.005	0.050	Died, 10 minutes	
21	0.005	0.050	Slight	
	Casein	Alcohol soluble protein		
22	0.005	0.050	Moderate	Not protected
23	0.005	0.050	Died	
24	0.001	0.050	Slight	
25	0.001	0.050	0	
26	0.005	0.050	0	
27	0.005	0.050	Moderate	
	Alcohol soluble protein	Milk		
28	0.003	3 c c	0	Protected
29	0.003	3 c c	0	Protected
30	0.003	3 c c	Died, 20 minutes	
31	0.003	2 c c	Severe	
32	0.004	2 c c	Severe	
33	0.004	2 c c	Severe	
	Milk	Alcohol soluble protein		
34	0.4 c c	0.050	Slight	
35	0.4 c c	0.050	0	
36	0.1 c c	0.050	0	No protection
37	0.1 c c	0.050	0	
38	0.2 c c	0.050	Slight	No protection
39	0.2 c c	0.050	Died, 40 minutes	
40	1.0 c c	0.050	0	
41	1.0 c c	0.050	Slight	
	Alcohol soluble protein	Lactalbumin		
42	0.005	0.050	0	
43	0.005	0.050	0	
	Lactalbumin	Alcohol soluble protein		
44	0.005	0.050	0	
45	0.005	0.050	0	
	Alcohol soluble protein	Lactoglobulin		
46	0.005	0.050	0	
47	0.005	0.050	0	
	Lactoglobulin	Alcohol soluble protein		
48	0.005	0.050	0	
49	0.005	0.050	0	
	Alcohol soluble protein	Gliadin		
50	0.005	0.050	0	Not protected
51	0.005	0.050	0	
	Gliadin	Alcohol soluble protein		
52	0.005	0.050	0	
53	0.005	0.050	0	
	Beef serum	Alcohol soluble protein		
54	0.5 c c	0.050	0	
55	0.5 c c	0.050	0	
	Alcohol soluble protein	Beef serum		
56	0.005	0.5 c c	0	
57	0.005	0.5 c c	0	

Experiments 1 to 17 show that lactoglobulin is an active anaphylactogen. The experiments with casein and lactalbumin are the same as those in previous tables and have already been discussed. The individuality of lactoglobulin and the alcohol soluble protein of milk is established by experiments 34 to 37. Experiments 38 to 49 show that lactoglobulin differs from the other 3 protein fractions of milk, since even in 1 mg. doses it sensitizes effectively to beef serum, and also intoxicates animals sensitized with small amounts of beef serum, corroborating the results obtained with the complement-fixation reaction by Bauer and St. Engel.[26] We thus find that the earlier observations on the capacity of beef serum and cow's milk to sensitize to each other is due solely to the globulin of the milk. This fact is in gratifying agreement with the chemical observation of Crowther and Raistrick since it indicates that bovine lactoglobulin is chemically identical with the globulin fraction of beef serum, although lactalbumin and serum albumin appear to be chemically different proteins.

The observation of Heuner that colostrum is biologically more closely related to serum than later milk, harmonizes with the high globulin figure obtained in colostrum by Crowther and Raistrick. The fact that colostrum contains large amounts of globulin, that this globulin seems to be identical with serum globulin, and that the serum globulin fraction carries the antibodies of the blood, is in interesting agreement with observations that have been made which indicate that the suckling animal secures important accessions to its antibody defense during the first days of life.[48]

The previously reported observations that cow milk and bovine serum are uncertain in their capacity to sensitize to one another is explained by the presence of a very small amount of globulin in milk, corresponding immunologically to serum globulin, for it has been shown by Julian H. Lewis [49] that a small amount of one antigen injected together with another antigen in excessive amounts, may be completely or partly prevented from manifesting its antigenic activity. This probably explains the fact that in our experiments, 0.1 c c of beef serum sensitized more effectively than 1 c c.

We have discussed, in connection with the other tables, the complete differentiation of alcohol soluble protein of milk from the three other protein fractions. It is to be recalled that this protein has only recently been described by Osborne and Wakeman, and that the application of the anaphylaxis reaction played an important part in the differ-

[48] See Reymann: Jour. Immunol., 1920, 5, p. 227.
[49] Jour. Infect. Dis., 1915, 17, p. 241.

entiation from the other proteins of milk. When large quantities of freshly precipitated casein were extracted at room temperature with 50-70% ethyl alcohol, it was found that the alcohol had dissolved out a considerable quantity of protein. As hitherto the only known alcohol-soluble proteins were of vegetable origin, it was suspected that this material might be either a cleavage product or a derivative of the casein. The anaphylaxis test demonstrated that it was not a proteose or similar cleavage product, since these do not exhibit active anaphylactogenic properties as does this alcohol-soluble protein (exper. 1 to 15), while cross sensitization as well as chemical tests showed that it was not casein. Further chemical study showed that it yielded a different proportion of some of the amino acids than did casein and was, therefore, a newly recognized constituent of milk. Although resembling gliadin in solubility, it is chemically distinct from this protein and in agreement therewith yields negative results with cross sensitization tests with gliadin (exper. 50 to 53). The positive cross experiments with milk indicate that it is a constituent of milk, and not formed by manipulation during isolation (exper. 28 to 41). With such a relative excessive proportion of other proteins present in the milk, it is not to be expected that effective sensitization with milk can be obtained. The amounts of the alcohol soluble protein which give strong sensitization to milk are so small (3 to 4 mg.) that sensitization with admixed casein seems to be excluded, since less than 1 mg. doses of casein sensitize but little.

Experiments 54 to 57 indicate that beef serum does not contain the alcohol-soluble protein of milk.

SUMMARY

Cow's milk contains 4 chemically distinct proteins or protein fractions, namely, casein, lactalbumin, lactoglobulin and an alcohol-soluble protein. By means of the anaphylaxis test it can be shown that these 4 proteins are immunologically distinct. This fact furnishes another striking illustration of the dependence of immunologic specificity on chemical composition rather than biologic origin. Of these 4 proteins only one, the globulin, sensitizes to beef serum or causes reactions in animals sensitized to beef serum. This corresponds to the observation of Crowther and Raistrick that lactoglobulin and serum globulin are chemically indistinguishable. That some positive cross sensitizations may be occasionally obtained between cow's milk and beef serum is explained by the fact that the globulin constitutes a very small

part of the milk proteins. Several other protein fractions obtained in studying milk proteins were, according to anaphylaxis tests, not distinct from the 4 known proteins of milk. Our experience with milk proteins, as well as with proteins of other sources, has demonstrated that immunologic methods are a great aid and in many cases indispensable in preparing proteins in a state of purity, and may be used to furnish information concerning chemical relations of proteins from different sources.

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PRIMARY SPONTANEOUS SQUAMOUS CELL CARCINOMAS IN MICE

STUDIES ON THE INCIDENCE AND INHERITABILITY OF SPONTANEOUS TUMORS IN MICE

FIFTEENTH COMMUNICATION

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Throughout most of the animal kingdom primary carcinoma of the skin is met with but rarely, in contrast with its great frequency in man, and also with the relative frequency of other cutaneous and subcutaneous tumors in animals. The existing literature on spontaneous tumors in animals indicates that no species except dogs, horses, and fowls, show skin carcinoma except as a rare condition, and this is of particular significance in view of the fact that skin carcinomas are more certain to be recognized than those in any other location and hence their relative infrequency is even more marked than any statistical evidence would indicate. Presumably the unprotected condition of the human skin accounts for its susceptibility to cancer, as the hairy scalp rarely shows primary carcinoma, and the pigmented skin of the negro also seems insusceptible.

The classical compilation of Sticker (1), which contains records of many tumors not fully differentiated according to modern criteria, gives the following relative frequency in this material of skin tumors in different species:

	SPECIES				
	Horse	Cattle	Dog	Cat	Swine
Total carcinomas	332	78	766	21	12
Skin	22	2	166	6	2
Eye	14	2	8		
Lip	4	1			
Anus	8		89	1	
Penis	52	2	16	1	
Vulva	11	1			

The extensive compilation by Teutschlaender (2) of the distribution of tumors in animals shows that cases of squamous cell carcinoma have been described in but few species, according to his records, namely: cat, dog, mouse, rat, elephant, beef, sheep, pig, horse, mule, cockatoo, toucan, chicken, carp, and gold fish. However, in another table he mentions also a case in a stag.

Apparently birds develop cutaneous carcinoma somewhat more often than most other animals, but even here there are but few recorded. In their summary of the literature on tumors in birds, Joest and Ernesti (3) found but 4 of 37 tumors described in birds to be skin carcinomas, those being two found on the legs of chickens by Wernicke; one on the wing of a "papapeis"—reported by Guerrini, and one on the wing of a toucan reported by Herbert Fox (4), this last case exhibiting also a metastasis in the lung. In none of their own 50 cases of tumors in birds was a skin carcinoma described. This compilation, as well as all others that we have observed, omits the striking case of squamous carcinoma of the skin of the foot of a hen described by Boynton (5), in which transplantations were made without success. More recently a similar tumor arising on the thumb claw of a lark has been reported by Urra (6). Two cases of squamous cell carcinoma of the mouth in fowls have been described by Pick and Koch. The experience of Teutschlaender (7) is unique, for he reports that of 54 carcinomas in fowls observed in the cancer laboratory at Heidelberg, 28 were skin epitheliomas, of which 18 were on the foot, attributed to the irritation of parasitic infection of the epidermis (*Cnemidocoptesmilben*).

Apparently fish are subject to skin tumors of various structure, including carcinoma. Thus, Fiebiger (8) reports cases of epithelioma occurring on the lip of each of two "Schlei" (*Tinca vulgaris* Cuvier) coming from the same pond, a markedly malignant skin epithelioma in a carp, and mentions papillomatous growths in several specimens of Kletterfish (*Anabus scandens*). Plehn has also described numerous skin epitheliomas in cyprinoid fishes.

Bashford has reported a squamous cell carcinoma in a small fish, *Gasterosteur spinachia*, two cases of carcinoma of the skin in frogs, and a carcinoma of the skin glands in a Triton.

Among mammals the dog alone seems to be commonly subjected to skin carcinoma, if we except the carcinoma of the penis and prepuce occurring not infrequently in horses and oxen. It would seem to be not uncommon in cats according to the experience of Roffo (9), who found 7 tumors in 307 cats examined, of which 4 were skin carcinomas on the head and face, with lymph gland metastases, but none of 11 cases of tumors in cats collected by Bashford (10) was of this type, and but 2 of 48 tumors in dogs, although there were several squamous cell growths in the mouth and pharynx. Leo Loeb (11) reported in 1903 that from one to three carcinomas of the inner canthus of the eye were observed every week in the Chicago stock yards, but in one year no other external carcinomas were observed except two of the vulva.

Rodents seem to have skin carcinoma but rarely. The large series of wild rats autopsied in plague work and examined for tumors (McCoy, Woolley and Wherry, Beatti) have revealed no such growths except for an epithelioma of the vulva reported by Woolley and Wherry (12). It will be recalled that the early transplantation experiments of Hanau (13) were with a squamous cell carcinoma from the vulva of a white rat, and he states that there had been two previous cases of similar growths in rats in their laboratory. Roffo (14) has also observed a squamous cell carcinoma which arose in the vulva of an old white rat, which was transplanted through six generations without change in structure. In view of the great frequency of parasitic skin infection in rats,

"rat scabies," commonly with extreme papillomatous proliferation of the epithelium (15), it is strange that epithelioma has not been more frequently observed. Of 123 cases of spontaneous rat tumors reported in the literature and collected by Bullock and Rohdenburg (16), there were but three epitheliomas, one of the tongue and two of the vulva. They reported on 48 rat tumors observed in the Crocker Laboratory, one of which was an epithelioma of the head, which was not described further. Morris (17) has reported a basal cell carcinoma which arose in the skin of a male rat, five months old, and which died out after two generations of transplants. He was unable to find other recorded instances of basal cell carcinomas in animals.

Several instances of squamous cell carcinoma have been described in mice, but they form an insignificant proportion of the many spontaneous tumors that have been observed in this species. Nevertheless they were looked upon as of great significance in the early days of experimental cancer research, since they established the fact that mice had other forms of tumor than the predominating mammary gland carcinoma. Haaland (18) reported a case of squamous cell carcinoma arising in the mouth of a mouse in Borrel's laboratory, involving the lower jaw, showing a typical squamous cell structure. Six mice were inoculated from this, but as in six months they showed no growths they were put back into the cage with the others. Ten months after the inoculations "deux cas identiques de cette même tumeur apparurent en même temps dans cette cage," but it was not known whether these were inoculated mice or not and Haaland does not make clear whether these later tumors were in the mouth, although this is apparently what he means. Later he observed a third case of mouth cancer in a mouse recently inoculated with a Jensen tumor. No growths developed in mice inoculated from this mouth tumor.

Murray (19) in 1908 described the following cases of superficial squamous cell carcinoma in mice: (1) A prickle cell growth with apparently little keratinization, arising in the skin of the neck, with metastasis into an adjacent lymph gland. (2) A carcinoma presenting both alveolar and squamous cell areas,

with keratinizing metastases in the lung. Presumably this tumor arose in the mammary gland. (3) Prickle cell growth without keratinization, interpreted as primary in the nipple; this mouse also had had a hemorrhagic adenocarcinoma of the mammary gland removed by operation. In discussing the mammary gland tumors he also describes the occurrence of areas of keratinization within tubular carcinomas, and regards them as indications of the close association of the mammary apparatus with the skin from which it develops, and points out that the ampulla which receives the terminal portions of the mammary ducts is also lined by stratified squamous epithelium. "Therefore, should the cells of the new growth have taken their origin near the nipple, variations in either direction are only to be expected."

In his later report from the London laboratories, Haaland (20) states that of 353 spontaneous tumors observed (not including those in Murray's report), 22 were squamous-celled carcinomas with marked keratinization, of which 14 arose in the mammary region and 8 outside it. Of these 8, 3 were mouth tumors similar to those observed in Borrel's laboratory, apparently arising in the mucous membrane of the inside of the cheek, there being in one metastases in the lymph nodes. One seemed to have arisen on the surface of the head, and it penetrated the skull; grafts from this were successful. (The illustrations of these tumors show the gross relations of such tumors so well that we are spared the necessity of reproducing further illustrations.) Three tumors were described as superficial verrucous growths, on the vulva, anus, and left flank. One was a typical squamous cell carcinoma of the skin of the chest, developing as a superficial ulcerating growth, "undoubtedly from the skin itself, or from the mammilla." A case of mixed squamous and sebaceous carcinoma of the vulva is also mentioned.

Several of the keratinizing mammary tumors differ from typical squamous cell carcinomas, and their cutaneous origin is doubtful. Four are described as resembling "molluscoid" tumors, characterized by long radiating cylinders of cells with a central keratinized zone, found in the mammary region and not giving successful transplants. Four tumors are described as "typical

squamous-celled alveolar carcinomata in the mammary region." Six others are called "adenocancroids," these being unquestionably mammary gland adenocarcinomas in which areas of keratinization occur. Attention is called to the occurrence of squamous-celled cysts in the normal mamma, apparently arising from mammary ducts in which the epithelium has become metaplastic, and which perhaps explains the occurrence of keratinizing carcinoma in the mammary gland. As squamous-celled growths have also been found rarely in the human breast, as well as in cats and dogs—Teutschlaender (21)—the disease in mice is not without analogy, although apparently relatively frequent among the mammary gland tumors of this species. Presumably in some stocks of mice keratinization is even more common in mammary gland carcinoma than the above reports indicate, for Woglom (30) found this change in 228 of 1000 spontaneous mammary gland carcinomas examined in the Crocker Laboratory.

Pick and Poll (22) reported as a "sweat gland carcinoma" a tubular growth arising near the scapula of a mouse, but the description published does not permit the exclusion of a mammary gland origin.

Erdheim (23) described a pedunculated tumor, which gave no evidence of malignancy, but exhibited a histological structure resembling squamous cell carcinoma. It arose from the vulva of a mouse, and after the outer part of the growth had been removed the base healed spontaneously. He was unable to classify this tumor, beyond indicating that it was a stratified epithelial neoplasm.

Tsutsui (24) has produced carcinoma on the skin of the back of mice by painting with tar, after the procedure used by Yamagiwa with the ears of rabbits, observing 16 carcinomas and 1 sarcoma in 17 mice surviving over 100 days. In two cases lung metastases were observed.

We find no other reports of squamous cell carcinoma arising in the skin or mouth of mice, which indicates their relative infrequency, since such tumors are most obvious when they do occur. Thus, Tyzzer (25) in his report of 83 spontaneous

tumors in mice includes no tumors of this type. In the 41 primary mouse tumors described by Jobling (26) there were no skin epitheliomas, although there was one of the molluscoid keratinizing tumors such as Murray, Haaland, and Tyzzer have described.

Squamous cell carcinomas outside the mammary gland and skin of mice are rare. Tyzzer reported that among his lung tumors "in several cases" the growth was of an epidermoid character, and Haaland described one such tumor. In our previous paper on primary carcinoma of the stomach in mice (27), we collected four cases of squamous cell gastric carcinoma reported in the literature and added three more in our own material, as well as one case of squamous carcinoma in the external surface of a chronically prolapsed rectum. Fiebiger (31) has also reported the occurrence of a few instances of squamous cell carcinoma produced experimentally in the stomach of mice by feeding cockroaches injected with Spiroptera. We have found no reports of squamous cell carcinomas arising in the esophagus or urinary tract, or in the cervix uteri, or in any other structure where they might be found in mice, except such as have been mentioned above.

OBSERVATIONS ON SKIN TUMORS AND SQUAMOUS CELL CARCINOMAS IN THE SLYE STOCK OF MICE

In 28,000 consecutive autopsies performed on mice of this stock, which had been permitted to live as long as possible without any experimental manipulations whatever, we have observed the following instances of primary neoplasms of squamous or stratified epithelial structure.

Primary squamous cell carcinoma of the skin and mouth	70
Primary basal cell carcinoma of the skin	15
Primary keratinizing carcinoma of the mammary gland	56
Primary squamous cell carcinoma of the stomach	4
Primary keratinizing carcinoma of the lung	1
Primary squamous cell carcinoma of the rectum	2
Primary squamous cell carcinoma of the vagina	1
Primary stratified carcinoma of the meibomian gland	2
Primary sebaceous gland adenocarcinoma	1
	152

As we have not yet made a complete analysis of all the tumors observed in these 28,000 autopsies the proportion of stratified cell tumors to total tumors cannot be stated. Roughly there are probably about 4000 primary spontaneous tumors of all sorts, so that these squamous cell tumors constitute not far from 4 per cent of the total.

CARCINOMA OF THE SKIN

In our material are 85 malignant epithelial growths arising in the skin, mouth, and lips, excluding those squamous cell growths that seem to be derived from mammary gland tissue and stratified cell growths from other glands. Of these, 70 are of squamous cell type and 15 of basal cell character. Since many of the tumors which arise about the lips and mouth are, when observed, so extensive that their exact point of origin cannot be determined, we have grouped them together with the skin tumors. Roughly classifying the site of the 70 squamous cell growths, 13 were in the skin of the trunk, one on a front limb, 2 on the vulva, 15 about the lower jaw, 18 on some other portion of the face, and 23 about the ears or neck; that is, all but 16 of the 70 were on the head and neck. Presumably this is to be explained by the much greater amount of traumatism suffered by the skin in this part of the body, which is especially marked in cage mice which are always rubbing their muzzles against the rough wire meshes of the cages. The tumors of the jaw and mouth in several instances seemed to have resulted from the irritation produced by broken or protruding teeth. Nearly all of the carcinomas arising on the trunk were definitely located at the site of a healed wound, and in not a few of those of the head the same origin was observed; it is probable that less obvious wounds are responsible for many if not all of the others. A chronic dermatitis often preceded the carcinomas of the skin of the face, presumably incited by traumatism. The not uncommon fungus infections of the skin seem to be too rapid in their course to lead to carcinoma formation.

Clinically these growths are similar to corresponding forms of carcinoma in man (fig. 1). The age at which they occur is, on an average, later than with any other mouse tumors, as they rarely appear before late middle life, and usually only in old age. They arise under crusted ulcers, or develop in areas of hyperplasia from chronic irritation, spread slowly as crusting, ulcerated growths, destroying at times the eyes or other features, usually remain superficial, and most often cause death by chronic infection of the ulcerated surface, which may also lead to acute



FIG. 1. HEAD AND FACE OF MOUSE WITH EXTENSIVE BASAL CELL CARCINOMA OF THE SKIN, OF AT LEAST FIVE MONTHS DURATION. NO. 7874

abscess formation. Occasionally death results from starvation when the cancer involves the mouth or jaw, from hemorrhage, invasion of the skull, or other coincident diseases.

The microscopic diagnosis of the skin neoplasms is often difficult, especially with the basal cell growths, since we find all stages of epithelial overgrowth from simple hyperplasia to metastasizing carcinomas. In this series no growth is included as a carcinoma unless it exhibited both the gross and clinical features of malignancy together with distinct microscopic evidence of infiltrative character. The extensive destruction of these growths by infective processes often obscures the microscopic findings. In several instances a growth that presented

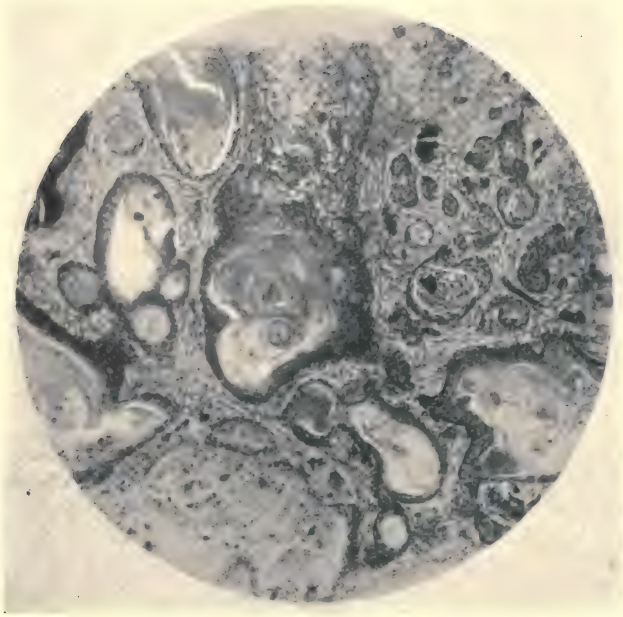


FIG. 2. TYPICAL SQUAMOUS CELL CARCINOMA OF THE JAW, WITH FORMATION OF ABUNDANT EPITHELIAL PEARLS. NO. 23331. × 60

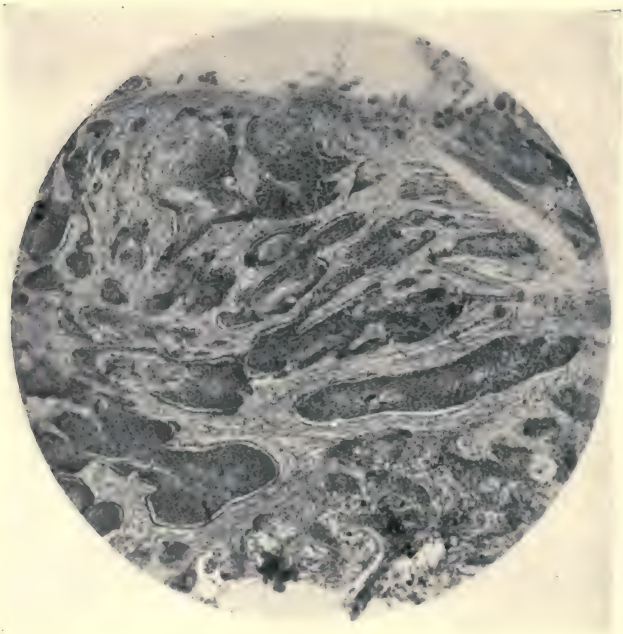


FIG. 3. SQUAMOUS CELL CARCINOMA OF THE JAW INFILTRATING ADJACENT TISSUES EXTENSIVELY. TYPE WITH LITTLE HORNIFICATION. NO. 26238. × 60

all the clinical features of carcinoma has been found at autopsy so extensively necrotized and suppurating that only examination of numerous sections from different parts of the growth has established the diagnosis of carcinoma. It is quite possible that we have lost a few genuine carcinomas through such destructive processes, to say nothing of cases in which the cannibalistic mate has selected the neoplasm for the first course.

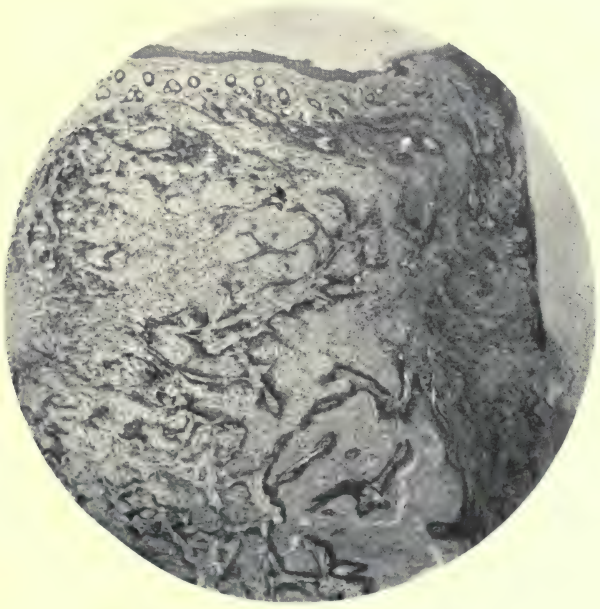


FIG. 4. SQUAMOUS CELL CARCINOMA OF SKIN OF CHEST ARISING IN THE SCAR OF AN OLD WOUND, THE SCAR TISSUE BEING ABUNDANT IN THIS FIELD, AND THE RELATION OF THE CANCER GROWTH TO THE OVERLYING SKIN BEING SHOWN. NO. 8212. × 45

The microscopic features of the squamous cell carcinoma the skin and mouth of mice differ not at all from those that occur in man. Usually hornification is marked (fig. 2), but, as in man, growths from a similar origin may show little or no tendency to form keratin (fig. 3). Usually the amount of connective tissue formation is not large, except in some cases where the cancer has developed in scar tissue from old wounds (fig. 4). Little help in diagnosis is afforded by mitotic figures,

since these are scanty in even the most typically malignant growths, and commonly cannot be found at all. Presumably they would be much more numerous if the tumors were removed from the living animal.

The infiltrative character of growth is not usually so extensive as we are accustomed to see it in man, for the mice generally succumb to infection from the ulcerated growth while it is

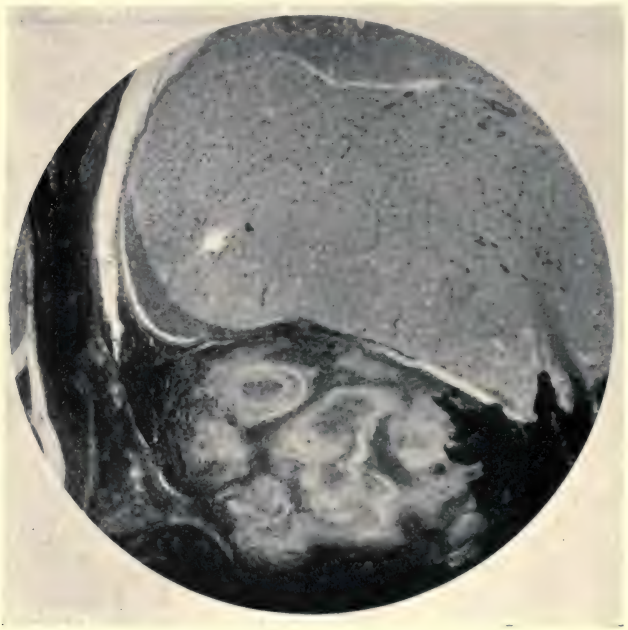


FIG. 5. SQUAMOUS CELL CARCINOMA OF THE HEAD AND FACE INVADING THE SPINAL COLUMN IN THE CERVICAL REGION, COMPRESSING THE SPINAL CORD AND INFILTRATING THE MENINGES
This tumor also invaded the skull. No. 23766. × 60

still small. Infiltration is usually seen best in the tumors that arise about the jaws, for here the bone is often invaded. Bone infiltration was observed in 7 of the 15 tumors that arose about the jaw. We have had one striking case (23766) in which a carcinoma of the skin of the head, beginning at the base of the right ear, infiltrated the skull and cervical vertebral canal, infiltrating the meninges about the cerebellum and compressing the spinal cord in the cervical region (fig. 5). This case resembles

the one described and pictured by Haaland, in which the skull was invaded. In two instances we have seen infiltration of the salivary glands, and in three cases an adjacent lymph node was involved apparently by direct extension.

The absence of lymphatic metastasis is a striking feature of these tumors when compared with corresponding growths in man, which is true of all forms of carcinoma in mice. In only two cases did we find a secondary growth by metastasis into a lymph node from a skin carcinoma (7950, 15232), and it was a very common observation that lymph nodes immediately adjacent to or in contact with these squamous cell growths, were not involved. Possibly serial sections of all our mice would have revealed other instances of metastasis, but the value of the information did not seem commensurate with the labor involved. In only one case did we find visceral metastasis (12627). This mouse had a squamous cell carcinoma arising just dorsal to the rectum, and a typical secondary nodule about 1 mm. in diameter in the lung; it also had a tubular carcinoma of the mammary gland which had produced no metastasis. The infrequency of metastasis in this series of spontaneous squamous cell carcinoma is significant when compared with Yamagiwa's experimental tar tumors in rabbits, since the infrequency of metastasis in his material has been thought by some to speak against their being true malignant tumors. As a matter of fact the metastasis incidence obtained by him, and by Tsutsui with experimental skin tumors in mice, is distinctly higher than that observed in these spontaneous growths.

The sex incidence is strongly in favor of the female (49 to 21) in this series. As most of the growths were on the head and neck we cannot account for this on the basis of erroneous inclusion of squamous carcinomas of the mammary gland with our skin tumors. It differs from our experience with other growths of non-reproductive tissues which have shown approximate equality as to sex incidence. Even more than with other tumors we have found that age is an important factor, these squamous cell carcinomas of the skin being predominatingly in old mice, and as many male mice die early from wounds received in fighting

this may account for the relative preponderance of females in this series.

Beyond the relatively slight extent of infiltration and metastasis these carcinomas present no noteworthy differences from human skin carcinomas. Often the amount of keratin scales piled up on the surface is strikingly great, and in one case the carcinoma arose at the base of definite cutaneous horns (25785). There is often a noteworthy amount of calcification of the necrotic scales. Cyst formation is frequently observed, and benign cutaneous cysts have been observed in several mice.

The basal cell carcinomas all arose on the face, ears, and head (fig. 1), 9 of the 15 being in females. Because these growths are of relatively low malignancy, as in man, they present particular difficulty in diagnosis, and we have set aside as "precancerous" numerous instances of basal cell hyperplasia of marked degree which did not present unqualified proof of malignancy. Quite frequently enormous hyperplasia of the cells about the hair follicles produces tumors of considerable size, and in these may be found areas highly suggestive of malignancy, in that altogether atypical plugs and masses of basal cells are formed, as shown in figure 6. Such growths, which very probably would have shown unqualified malignancy had the mouse lived longer, have not been included among the basal cell carcinomas. In general, basal cell carcinoma in the mouse corresponds entirely to the corresponding growth in man (fig. 7). To quote MacCallum (28), "In spite of the complexity of the downward growing strands all reach to about the same level. Further, it is seen that they are very sharply outlined against the stroma and show little inclination to strew their cells into the irregular crevices of that tissue." We have found the various modifications of basal cell growths commonly described, such as the formation of tubules suggestive of undeveloped hair follicles, hornifying surface areas with typical basal cell growths beneath, and the so-called adenoid epithelioma which seems intermediate between basal and squamous cell growths. Among our keratinizing carcinomas of the mammary gland was one of cells that suggested a basal cell character. No metastases were

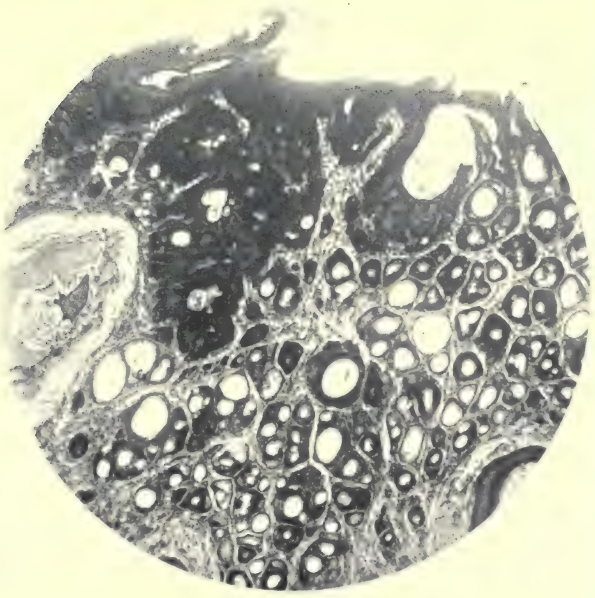


Fig. 6. Basal Cell Hyperplasia of an Irritated Muzzle, with Areas of More Atypical Growth

There have been numerous such growths, which are probably properly designated as precancerous, but may be actually malignant. No. 16563. × 60.

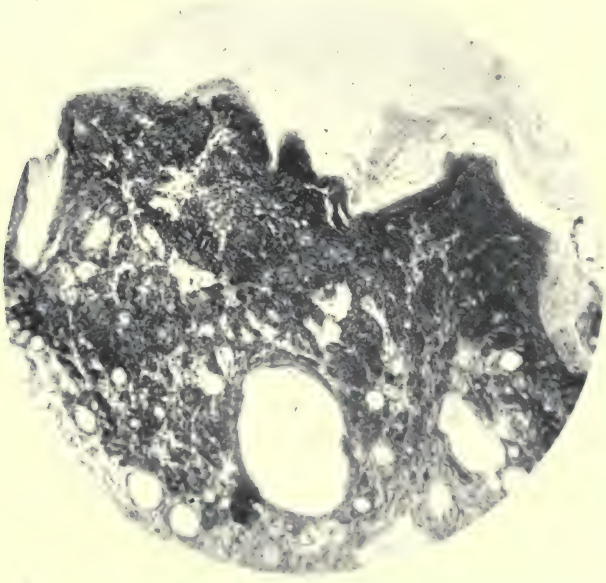


Fig. 7. Basal Cell Carcinoma Which was Highly Malignant, as It Entirely Replaced the Face, Destroying Both Eyes, and Forming Tumor Masses in the Orbits. No. 9932. × 90

observed among these cases, although in one, extensive infiltration destroyed both eyes and produced large tumor masses in the eye sockets. Several of these growths arose at the site of distinct scars from wounds.

Despite the fact that these skin tumor mice were all well advanced in age the co-existence of other tumors is lower than that seen in many other forms of mouse tumor. Perhaps one factor in this is that most of the mice with skin cancer had lived beyond the age at which other tumors occur most frequently. It also seems that the heredity of these mice is responsible to some extent, but a complete analysis of this factor has not yet been made. Of the 85 cases, in which 58 were females and 27 males, but 6 showed a carcinoma of the mammary gland, 7 exhibited lung adenoma (including one that also had a mammary gland carcinoma), one had an adenoma of the ovary and one seemed to have pseudoleukemia. Despite the number of old wounds in these mice no cases of sarcoma were observed, presumably because of either old age or ancestry, but one case of carcinoma arising in the mouth and infiltrating the jawbone, showed such a flattening of the deeper cells that for some time we were in doubt whether or not it was an instance of mixed sarcoma and carcinoma. This case (8560) is described and pictured in our article on sarcoma in mice (29).

SQUAMOUS CELL CARCINOMAS OF THE MAMMARY GLAND

The keratinizing carcinomas of the mammary gland form an interesting group, and properly lie outside the scope of this paper, except for the fact that with not a few of them it is extremely difficult to tell whether we are dealing with a primary mammary gland carcinoma or a skin carcinoma arising over the mammary gland. Three types of these tumors can be distinguished.

1. Carcinomas of the mammary gland which are essentially cylindrical cell carcinomas, forming tubules and alveoli, but some areas of which undergo a transformation into stratified epithelium with the formation of keratin, often in large amounts (fig. 8). These are the "adenocancroids" of Murray and

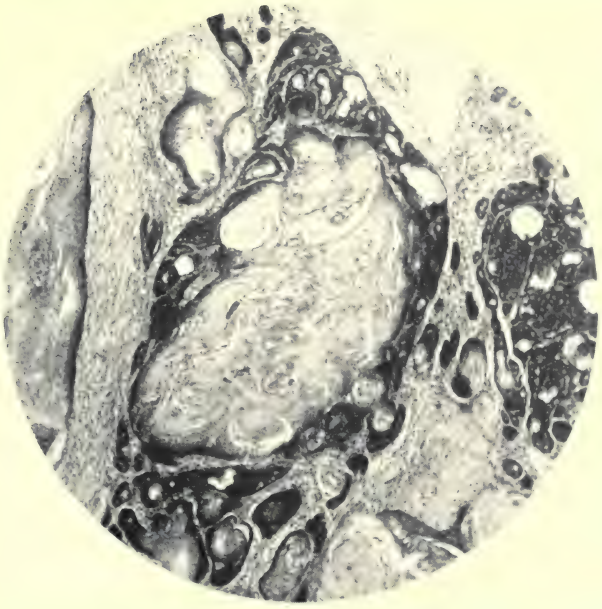


FIG. 8. TUBULAR ALVEOLAR CARCINOMA ("ADENOCANCROID") OF THE MAMMARY GLAND WITH AREAS OF KERATINIZATION

To the left is seen a band of densely keratinized tissue with no evidence of its glandular origin. No. 13336. × 60.

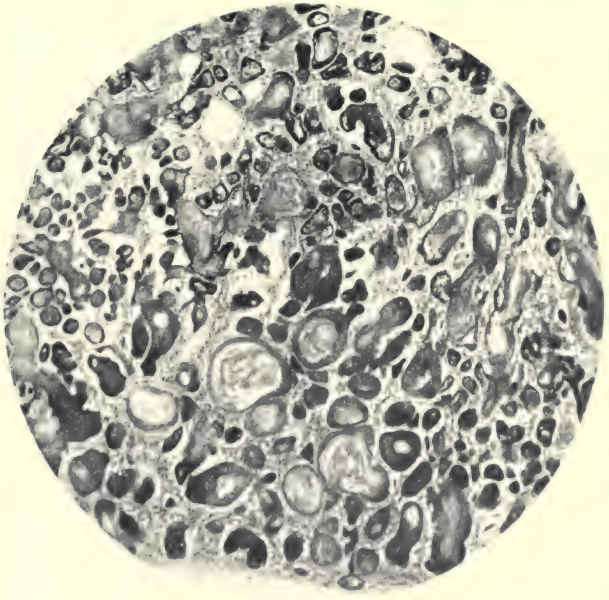


FIG. 9. SUPERFICIAL ADENOCANCROID, APPARENTLY ARISING IN THE DUCTS OF THE MAMMARY GLAND

Shows very little evidence of origin in glandular tissue. This mouse also had three other tubular carcinomas of the mammary gland without keratinization. No. 15300. × 60.

Haaland. It is probably of some significance that the keratinization is usually most marked in the portions nearest the cutaneous surface, as if it began in the ducts. The amount of keratinization varies, sometimes appearing in only a few small spots in the tumor tissue, but often extending until little of the original columnar cell type of tissue remains.

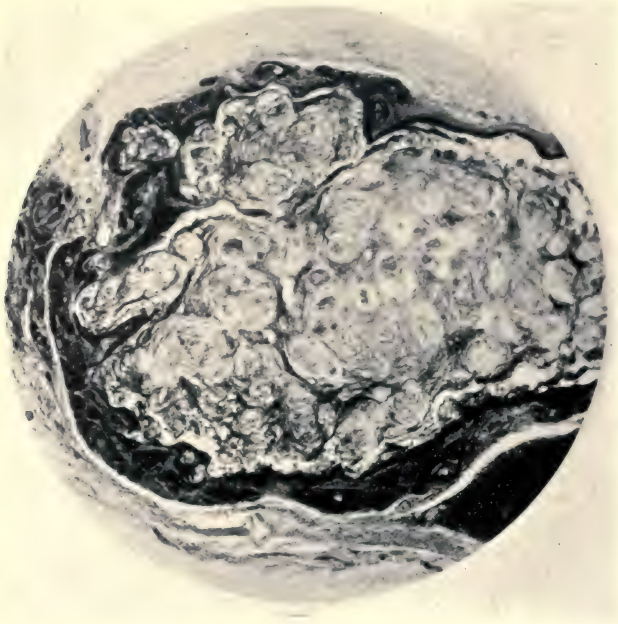


FIG. 10. KERATINIZING CYSTS IN THE MAMMARY GLAND

Such structures are not infrequently found, and may be the precursors of some of the malignant keratinizing carcinomas of the mammary gland. No. 8271. × 40.

2. Squamous-celled keratinizing carcinomas without any evidence whatever of cylindrical cell structure, but arising subcutaneously in the mammary regions (fig. 9). Many of these present no histological evidence that they are derived from the cells of the mammary gland, but they are observed to arise within the gland substance and sometimes are still entirely subcutaneous when the mouse dies. The occasional presence

within the mammary gland of what seem to be simple benign cysts of stratified epithelium with masses of desquamated hornified material (fig. 10) indicates the probable origin of such tumors. When they ulcerate on the surface it may be impossible to differentiate them from primary carcinoma of the skin or nipple, for histologically they differ little if at all from the usual squamous

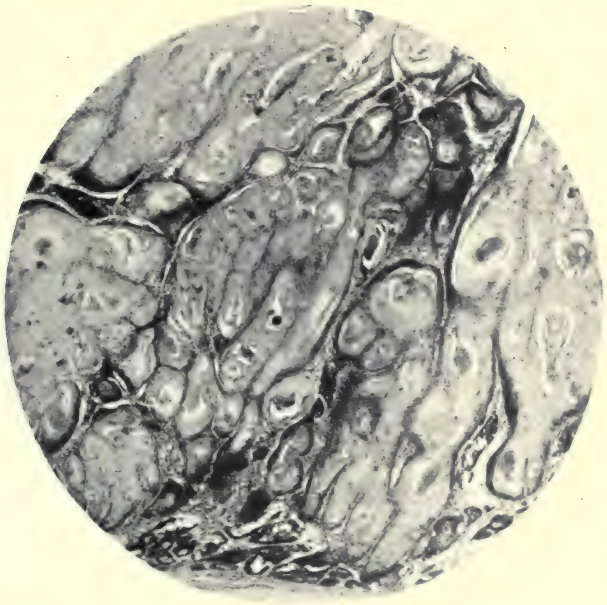


FIG. 11. MOLLUSCOID CARCINOMA OF THE MAMMARY GLAND

This growth, which arose in an inguinal mammary gland, invaded the abdominal wall and protruded into the pelvis, but produced no metastases. A nonkeratinizing carcinoma of the mammary gland was also present which produced pulmonary metastases. No. 15622. × 60.

cell carcinoma of the skin. When observed early their origin in the gland beneath the skin is usually the chief ground for recognizing them as mammary gland tumors; nevertheless, the fact that after differentiating, on a histological basis solely, the skin carcinomas from the mammary gland carcinomas we found that nearly all those we had selected as of cutaneous origin had

arisen at the site of old skin wounds, indicates that there are usually recognizable differences.

3. Molluscoid carcinomas of the mammary gland, to adopt Borrel's designation of these striking growths with long, radiating cylinders of stratified epithelium with a large central core of keratinized material (fig. 11). These seem to grow out from the tubules near the nipple, as if they arose from the stratified portion of those ducts. They produce ulceration of the surface about the nipple, which greatly enhances their resemblance to the skin carcinomas.

We have little to add to the statements of Borrel, Murray, Tyzzer, and Haaland concerning these squamous cell growths arising within or about the mammary gland, and the excellent illustrations accompanying Haaland's article make any further illustrations by us unnecessary. A few points may be worth recording. Forty-three of the 56 squamous-celled mammary gland tumors may be best designated as of the adenocancroid type, in that they show a greater or less proportion of cylindrical cell structure. Several of these were multiple, as is often the case with primary carcinoma of the mammary gland. In three there were two adenocancroids, one mouse had three, and one had five, four arising distinctly in mammary gland tissue while the fifth arose anterior to the urethra and seemed to have originated in the skin, and this showed no glandular elements. Fourteen mice had an adenocancroid and also one or more other independent mammary gland carcinomas that showed no keratinization. Of these, one (9365) exhibited also a typical squamous cell carcinoma metastasis in the lung; one mouse with three primary adenocancroids (5738) showed a lung metastasis while three mice that had each two mammary gland tumors, one of each type, had pulmonary metastasis of the cylindrical cell type. That is, from 43 cases of adenocancroid of the mammary gland, but one metastatic nodule of adenocancroid has been seen in the lungs, and none in the lymph nodes or elsewhere. Not a few of these mice had also co-existing tumors other than those of the mammary gland, namely, one with a squamous cell carcinoma of the muzzle (9962), one with a malignant ovarian tumor

(6801), two with uterine fibroids (14370 and 14811); one with a lung adenoma (12098); one with an adenoma of the liver and an adenoma of the lung (9544); one with an adenoma in the lung and a strange growth in a subcutaneous lymph node that might be interpreted as an endothelioma (15622) and one had an intrapelvic sarcoma with metastasis into the liver and also into an ovary which contained in addition a small adenoma (10006).

Two of the adenocancroids were examples of the not uncommon mixture of sarcoma and carcinoma in the mammary gland of the mouse (15412 and 6182).

Only four of our tumors were typical of the molluscoid cancers, although more or less of this character was shown by several of the growths diagnosed as adenocancroids. One of these four mice had also an adenoma of the ovary (496) and another had a benign lung adenoma (5581). None of these four keratinizing tumors produced metastases.

Nine squamous cell carcinomas that exhibited no glandular structure to identify them as mammary gland tumors, were believed to have this origin because they arose beneath the skin, and in most instances did not ulcerate through at any time. These growths, however, seem to be of a more malignant character than the adenocancroids; three of them produced squamous cell metastases. No. 10936, which is remarkable in being the only male with a subcutaneous stratified epithelial growth that seemed to arise in the mammary gland, exhibited squamous cell metastatic growths in the lung, in the mediastinum, and in the chest wall. Another (7950), with three subcutaneous, non-glandular squamous cell carcinomas, showed a metastatic nodule in one lymph node and direct infiltration of a second. No. 13671 with a subcutaneous squamous cell carcinoma and also a cylindrical cell mammary gland carcinoma, had three large metastatic nodules in the lungs all of squamous cell structure. As to multiplicity of tumors in this group there were three independent subcutaneous squamous cell tumors in each of two mice (2239 and 7905), two had a simple cylindrical cell carcinoma of the mammary gland in addition to the squamous cell growth (7526 and 13671), while one had adenomas of the liver and ovary

(14047) and one mouse had leukemia (8236). In this group we have included one squamous cell tumor (24858) that differs from the rest in having the non-keratinized tumor elements suggest strongly the appearance of a basal skin carcinoma, although the growth seemed to arise in the mammary gland.

SQUAMOUS CELL CARCINOMA OF STOMACH AND RECTUM

In a previous paper on primary carcinoma of the stomach in mice (27) we collected records of four reported cases of squamous cell carcinoma arising in the cardiac portion of the stomach to which we added three observed in this laboratory. Since that publication we have observed one more case (24367). This was an old female mouse that for some time before death had been isolated because suffering from tape worm infection. The stomach wall in the cardiac portion was much thickened and small white nodules were scattered over the outer surface. No metastases could be found. In the liver was an encysted cestode, and there was a prolapsed rectum showing much thickening of the wall. Microscopically the nodules in the stomach are of squamous cell carcinoma, apparently still quite early but of typical structure. One small nodule of similar structure is found in the omentum. The rectum shows a marked overgrowth of squamous epithelial plugs on the outer, ulcerated surface of the bowel, and with many of these an infiltrative character is suggested, but apparently this lesion cannot be diagnosed as carcinoma.

Another new case of carcinoma of the stomach (25911) is more difficult to classify. A female Peromyscus mouse, nearly four and a half years old, which had been isolated all her life and never bred, refused food for several days before death. At autopsy there was found a marked thickening of the entire stomach wall, particularly at the cardiac end where it seemed nearly solid from wall to wall. A thick white nodular outgrowth 6 by 4 by 4 mm. of similar tissue bound together the stomach, liver, and esophagus, while another nodule 8 by 4 by 4 mm. bound together the pancreas and intestinal mesentery posterior

to the stomach. No other metastases were found. Microscopically this growth is composed of groups of strands of infiltrating epithelial cells, which are not hornified, neither do they form tubules. They grow profusely throughout all coats of the stomach, sometimes forming sheaths about the tubules of the cardiac portion. The nodules outside the stomach are secondary growths of identically the same structure in lymph nodes. It is not possible to be sure whether this carcinoma is derived from the columnar or the stratified epithelial portions of the stomach. While the gross appearances suggested an origin in the cardia, which is the site of all but one of the recorded cases of gastric carcinoma in mice, yet all of these were frankly hornifying squamous cell carcinomas.

The full details of the three other cases of squamous cell carcinoma occurring in this series are published with illustrations in our previous communication (27) and need not be repeated here. To recapitulate them briefly they were as follows:

No. 5802, male two years old. Squamous cell carcinoma of pyloric portion of the stomach, measuring 15 by 15 by 12 mm. A secondary growth 11 by 12 mm. in the mesentery. No other metastases. Some infiltration of the pancreas.

No. 7851. Male, twenty-five months old. Cardiac portion of the stomach is ulcerated and thickened, forming a mass 16 by 12 by 8 mm. No metastasis. Structure, typical squamous carcinoma, infiltrating all coats of the stomach, and invading the adhesions between the stomach and liver.

No. 16440. Female, aged twenty-seven months. At the junction of the cardiac and pyloric portions a thickened mass from 3 to 15 mm. wide surrounds the stomach. Nodules were found in the omentum and mesentery, metastases in lymph nodes. Structure, typical squamous cell carcinoma with some hornification.

In the same paper was published with illustrations the report of a case of squamous cell carcinoma arising in the metaplastic epithelium covering a prolapsed rectum (8345). This was a male mouse, which had had a prolapsed rectum for six months before its death. The growth was not extensive but seemed to be

typically squamous cell carcinoma arising in metaplastic epithelium. Since then we have observed a second similar case.

No. 20052. Female, had a prolapsed rectum for four months before death, which was from hypertrophy of the heart and pulmonary edema. The surface of the prolapse became much ulcerated and after a time showed what seemed to be a proliferation of epithelium. The regional lymph nodes were somewhat enlarged. Microscopically the external surface of the prolapsed bowel shows, in addition to considerable ulceration, areas of definite squamous cell carcinoma, with strands of cancer cells infiltrating through a thick layer of granulation tissue down to but not into the muscularis. Apparently this has arisen from what was part of the cutaneous surface of the anus, and not from metaplastic mucosa as in the previous case. As a possible factor in the inciting irritation, a small piece of wood was found imbedded in the bowel wall, surrounded by granulation tissue and cancer cells.

CARCINOMA OF THE VULVA AND VAGINA

No cases of squamous cell carcinoma of the uterus have as yet been described in mice, so far as we can learn. Although we have found a few fibromyomas and sarcomas of the uterus, we have met with but one epithelial neoplasm, which was an adenocarcinoma.

Erdheim (23) and Haaland (20) each have described a recurrent verrucous growth of the vulva, and the latter a mixed squamous and sebaceous cell carcinoma of the vulva. Two cases of squamous carcinoma of the vulva have been observed in this series and are included among the 70 squamous cell skin carcinomas. (1) No. 7950. This occurred as a condylomatous growth arising distinctly in the vulva of an old mouse, infiltrating the subcutaneous tissues, and was an unquestionably malignant squamous cell growth microscopically. There was in addition metastatic growth in two lymph nodes in the groin. (2) No. 18928. A growth of a warty character and ulcerating slightly developed on the vulva some time before the death

of the mouse, spreading about the rectum before death. At autopsy there was found in addition an enlarged lymph node attached to the right ureter. Microscopically the external growth is a typical squamous cell carcinoma with much hornification, and not very marked tendency to infiltration. The nodule attached to the ureter was a lymph node containing a cyst lined with squamous epithelium, or, to describe it better, an epithelial cyst covered with a thin layer of lymphoid tissue. The epithelial wall of the cyst is thin, shows no evidence of infiltrative or other malignant character, and hence does not at all resemble a metastatic growth, but it is difficult to explain the presence of such a structure in this location on any other basis.

In addition to these two carcinomas we have observed an excellent case of carcinoma of the vagina.

Carcinoma of vagina (no. 22582). This mouse was found, nearly a month before its death, with a 3 mm. pink nodule protruding from the vagina. At the time of its death from pneumonia it presented an ulcerating mass 15 by 12 by 10 mm., about one-half of which protruded from the vulva. It seemed to arise from the vaginal wall, the uterus and bladder not being involved, but it ulcerated into the rectum and was much infected and ulcerated. No enlarged glands or other evidences of metastasis could be found. Microscopically the growth is composed mostly of loose masses of keratinized scales exfoliated from the underlying growth, which infiltrates the vaginal wall as a typical squamous cell carcinoma.

KERATINIZING TUMORS OF THE LUNG

As mentioned in our review of the literature, Haaland has described one case of primary keratinizing growth in the lung, and Tyzzer says that he has had "several cases." The influence of heredity on the incidence of tumors of special types is suggested by the fact that of several hundred cases of primary lung tumors in the Slye stock, but one has been definitely found to show keratinization.

No. 13314. A male, age twenty months, which showed no other autopsy findings of interest, had the lower lobe of the left

lung nearly replaced by a yellowish mass, which was distinguished from the ordinary papillary adenoma of the lung chiefly by its color. This made it resemble an abscess, but it was much too hard to be an abscess, and there was no pleural exudate or adhesion. Microscopically the tumor consists chiefly of a mass of hornified scales heaped up in waving, concentric layers. Only at the very periphery are living cells found. Here is a narrow growing border of stratified epithelial cells, differing in no essentials from that seen in epitheliomas of the skin. There is no marked tendency to infiltration, the growth apparently progressing by expansion, but the presence of occasional mitotic figures is noted. About the growth there is much round and spindle cell proliferation and numerous foreign body giant cells are found. Our specimen differs from the one illustrated by Tyzzer in having a smaller proportion of living cells. We are not certain whether this growth represents a true neoplasm or a progressive metaplasia due to some persisting chronic inflammatory condition, but the former seems more probable.

Two other mice have shown somewhat related pulmonary conditions.

No. 10561. This mouse, which had also a carcinoma of the skin, had a benign adenomatous growth in the lung which showed some tendency to stratification, but without keratinization.

No. 25136. A male mouse had in the right upper lobe of the lung a mass 14 by 12 by 10 mm. resembling in appearance a malignant tumor of the lung. Microscopically this tissue resembles much more closely an unresolved organizing and necrotizing pneumonia, in which are two irregular, independent areas composed of masses of keratinized scales, with a slender border of flattened epithelial cells. It is quite impossible to decide whether this is a true tumor, or whether it is the cause or the result of the pneumonic condition, although the epithelial growth has the appearance of being much older than the pneumonic process.

TUMORS OF THE MEIBOMIAN GLAND

Two mice have presented growths arising in the eyelids, which, according to their structure, seem to be adenomas arising in the Meibomian glands.

No. 18099. A small, slowly growing mass developed beneath the left eye of a female mouse; from the eye exuded a small amount of thick white exudate. The growth had reached a diameter of 10 mm. when the mouse died from an acute lung infection, and showed no evidence of infiltration or ulceration. Microscopically the growth is composed of papillary structures covered with many layers of epithelial cells. It differs from the normal Meibomian gland in the exaggeration and lawless arrangement of the structures, and the greatly increased number of epithelial cells covering the stroma. The diagnosis of benign adenoma of the Meibomian gland seems justified, especially in view of the size of the growth and the findings in the next case.

No. 27929. An old female mouse, which died of chronic nephritis, had a soft mass, 6 mm. in diameter, beneath the left eye. The gross appearance suggested an epithelioma. Microscopically this tumor is quite the same as the one described immediately above, except for the important fact that it infiltrates down to the bone of the orbit, thus indicating that it is a malignant infiltrating adenocarcinoma.

SEBACEOUS GLAND ADENOCARCINOMA

Such tumors have been described in mice by Murray, Tyzzer, and Haaland, the last two having transplanted them successfully. We have found one case of sebaceous adenocarcinoma of the preputial gland, which closely resembles the growth described and illustrated by Haaland (20).

No. 19895. Beginning at the base of the penis is a mass 30 by 25 by 25 mm. extending well into the inguinal region. The penis was completely imbedded in the tumor, the older portions of which were softened, but about the periphery were hard nodules of newer growth. The testicles and epididymis were

not involved. Death resulted from chronic nephritis. There were no metastases. Microscopically this tumor (fig. 12) reproduces closely the normal structure of the preputial gland, but not infrequent infiltration of the stroma by strands of epithelial cells corroborates the gross evidences of malignancy. It corresponds perfectly to the illustration given by Haaland, who also

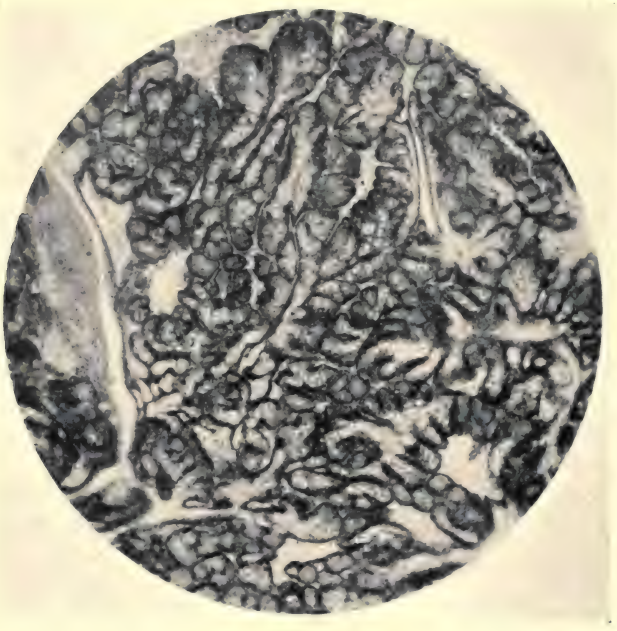


FIG. 12. SEBACEOUS ADENO-CARCINOMA OF PREPUTIAL GLAND

This was a large tumor with distinctly malignant characteristics, infiltrating the adjacent tissues, despite its close resemblance to the normal structure of the gland from which it arose. No. 10895. × 60.

comments on the close resemblance to the normal gland in spite of the definitely malignant character.

SUMMARY

Among 28,000 mice dying natural deaths at all ages, and carefully autopsied, have been observed 153 growths of stratified and squamous epithelium that correspond by the usual

standards to true neoplasms, excluding a considerable number of epithelial growths which lack positive conclusive evidence of neoplastic character, although possibly some of these are also early carcinomas. Seventy-one are examples of squamous cell carcinoma of the skin or mouth. They differ from the human skin carcinoma chiefly in a low incidence of metastasis. Fifteen others are of basal cell character, arose always about the head, and produced no metastases. In both these groups the incidence is higher in the females than in the males. Trauma and chronic irritation seem to play an important part in the production of skin carcinoma in mice, most of our cases occurring about the head and face, often recognizably at the site of wounds, and nearly all the skin carcinomas of the trunk arose in old scars. Skin cancers occur at a greater average age than other tumors in mice.

Fifty-six examples of squamous cell keratinizing growths arising in the mammary gland were observed, predominatingly adenocarcinomas with localized areas of keratinization. These also seldom produce squamous cell metastases.

Other tumors in this group were: Four squamous cell carcinomas of the stomach, two arising in the prolapsed rectum, two in the vulva, one keratinizing tumor of the lung, one sebaceous adenocarcinoma of the preputial gland, and, as hitherto undescribed mouse tumors, one squamous carcinoma of the vagina and two adenomas of the Meibomian glands, one of these being infiltrative and apparently malignant.

The literature of the comparative pathology of squamous cell carcinoma in animals is reviewed, and it is worthy of comment that as yet no cases of squamous cell carcinoma of the uterus, bladder, or esophagus seem to have been described in mice.

REFERENCES

(1) Sticker: Arch. f. klin. Chir., 1902, lxv, 616, 1023.
(2) Teutschlaender: Ztschr. f. Krebsforsch., 1920, xvii, 285.
(3) Joest and Ernesti: Ztschr. f. Krebsforsch., 1915, xv, 1.
(4) Fox: Jour. Path. and Bacteriol., 1912, xvii, 217.
(5) Boynton: Festschrift for Victor C. Vaughan, 1903, p. 535.
(6) Urra: Rev. de med. y cirug. práct., Madrid, 1918, cxviii, 321.

(7) Teutschlaender: Ztschr. f. Krebsforsch., 1920, xvii, 350.
(8) Fiebiger: Ztschr. f. Krebsforsch., 1909, vii, 165.
(9) Roffo: Rev. d. Inst. Bacteriol., Buenos Aires, 1918, 1, 333.
(10) Bashford: Third Sci. Rep., Imperial Cancer Research Fund, London, 1908, p. 41.
(11) Loeb: Arch. f. klin. Chir., 1903, lxx, 845.
(12) Woolley and Wherry: Jour. Med. Res., 1911, xxv, 205.
(13) Hanau: Fortschr. d. Med., 1889, vii, 321.
(14) Roffo: Rev. d. Inst. Bacteriol., Buenos Aires, 1918, i, 155.
(15) Lipschütz: Wien. klin. Wchnschr., 1920, xxxiii, 426.
(16) Bullock and Rohdenburg: Jour. Cancer Res., 1917, ii, 39.
(17) Morris: Jour. Cancer Res., 1920, v, 147.
(18) Haaland: Ann. de l'Inst. Pasteur, 1905, xix, 165.
(19) Murray: Third Sci. Rep., Imperial Cancer Research Fund, London, 1908, p. 69.
(20) Haaland: Fourth Sci. Rep., Imperial Cancer Research Fund, London, 1911, p. 1.
(21) Teutschlaender: Ztschr. f. Krebsforsch., 1920, xvii, 300.
(22) Pick and Poll: Berl. klin. Wchsnchr., 1903, xl, 518.
(23) Erdheim: Ztschr. f. Krebsforsch., 1906, iv, 33.
(24) Tsutsui: Gann, 1918, xii, 17.
(25) Tyzzer: Jour. Med. Res., 1909, xxi, 479.
(26) Jobling: Rockefeller Inst. Monographs, 1910, i, 81.
(27) Slye, Holmes and Wells: Jour. Cancer Res., 1917, ii, 401.
(28) MacCallum: Text-book of Pathology, Philadelphia, 1920, p. 1032.
(29) Slye, Holmes, and Wells: Jour. Cancer Res., 1917, ii, 1. Fig. 3.
(30) Woglom: Jour. Cancer Res., 1917, ii, 471.
(31) Fiebiger: Jour. Cancer Res., 1919, iv, 367.

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THE INFLUENCE OF HEREDITY IN DETERMINING TUMOR METASTASES[1]

STUDIES IN THE INCIDENCE AND INHERITABILITY OF SPONTANEOUS TUMORS IN MICE

SIXTEENTH REPORT

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Tumor metastases in this stock are somewhat rare. In 29,000 necropsies, furnishing something over 4000 primary spontaneous tumors, mostly malignant, only about 19 per cent of the tumors have metastasized. It has been the general opinion of pathologists that the formation of metastases is a distinguishing property of malignant tumors. The infiltration of tumor cells into tissues adjacent to the neoplasm, and the entrance of these cells into the lymph and the blood streams are the usual mechanical bases for secondary growths. But all these conditions exist in innumerable instances, both in human and in animal tumors, without the occurrence of secondary growths. For example, females 7821 and 9260 of this stock (see chart 11, strain 508, p. 158) each had a large and highly malignant infiltrative mammary gland carcinoma of relatively long standing. In both instances there were multiple tumor emboli in the lungs, but no secondary growths arose from these emboli,—i.e., the mechanical basis was there in each case, the occurrence of tumor did not follow.

[1] Presented before the American Association for Cancer Research, Cleveland, Ohio, March 24, 1921.

Ewing (1), in his chapter on metastasis, speaks of "a peculiar susceptibility of some tissues to develop secondary tumors," but later states that "the mechanism of circulation will doubtless explain most of these peculiarities, for as yet there is no evidence that any one parenchymatous organ is more adapted than others to the growth of embolic tumor cells." There would seem, then, to be some factor, other than the mechanical one, which determines the incidence and location of metastasis, even though it be minimized, as Ewing has done in the paragraph cited.

Again, Ewing states that in highly vascular tissues like the lip, stomach, and testicle, very small carcinoma may yield distant metastases. But these very organs are among those whose tumors rarely metastasize in this stock. For example, among the lip carcinomas in this stock none has metastasized; among the testicle tumors (2), one metastasized locally, while five out of six stomach carcinomas (3), (4), showed metastases in regional lymph-nodes only, but no vascular metastases in the liver or elsewhere. And this in spite of the close resemblance of these tumors to human tumors of similar type in similar organs.

In this stock, mammary gland carcinoma and sarcoma rarely metastasize into regional lymph-nodes, in marked contrast to human breast tumors of the same type. Indeed, in this stock, mammary gland tumors can be seen growing up to the lymph-node but not invading it, even by extension. On the other hand, pulmonary metastases from mammary gland carcinoma and sarcoma are common in this stock, again in contradistinction to the reported behavior of human breast tumors of the same types.

These divergencies (which are typical of the many divergencies) in the metastasis behavior of tumors of similar types and in similar organs, require some explanation other than merely the mechanical tendency of certain types of tumors to metastasize in certain locations, and that there is some other factor is generally recognized by pathologists, although little progress has as yet been made in demonstrating what this factor is.

The matter of tumor metastasis in this laboratory has been under observation for twelve years, and the data here given are based on over 29,000 necropsies, among which every instance of metastasis is known. The charts used to demonstrate the points made in this report are perfectly typical. The non-tumor fraternities (where there are any) have been omitted, in order to save space and to show exactly where these tumors occurred.

It must be borne in mind in reading these charts, that, as I have previously stated, tumor metastases in this stock are rather rare, being only about 19 per cent of the primary malignant tumors. A given chart then, which shows many primary tumors, must not be expected to show large numbers of secondary tumors. For example, a chart showing 8 primary tumors, would, if this general average of 19 per cent were carried out, show only 1.5 cases of metastasis, etc. The biological evidence from this laboratory, which explains this low percentage of metastasis from malignant tumors, will be given further on in this report.

From this study three points stand out with striking clearness:

POINT I

In any given strain the metastatic tumors (where there are any) tend to occur in exactly the same organs in which the primary tumors of that strain occur.

Note chart 1, showing part of strain 215 and some of its derivatives. The parent female, no. 3, had a sarcoma-carcinoma of the mammary gland, a malignant adenoma of the liver, and a metastatic sarcoma of the kidney. The parent male, no. 360, came from a strain carrying lung and mediastinal tumors, and he himself was proved heterozygous to tumors in these locations (i.e., he carried them potentially and introduced them in every strain into which he was crossed, although he did not himself show these tumors).

Note here how these particular types and locations of tumors have "segregated out" and certain of them have been transmitted to the branches of the strain here shown, both as primary

PART OF STRAIN 215 AND SOME DERIVATIVES

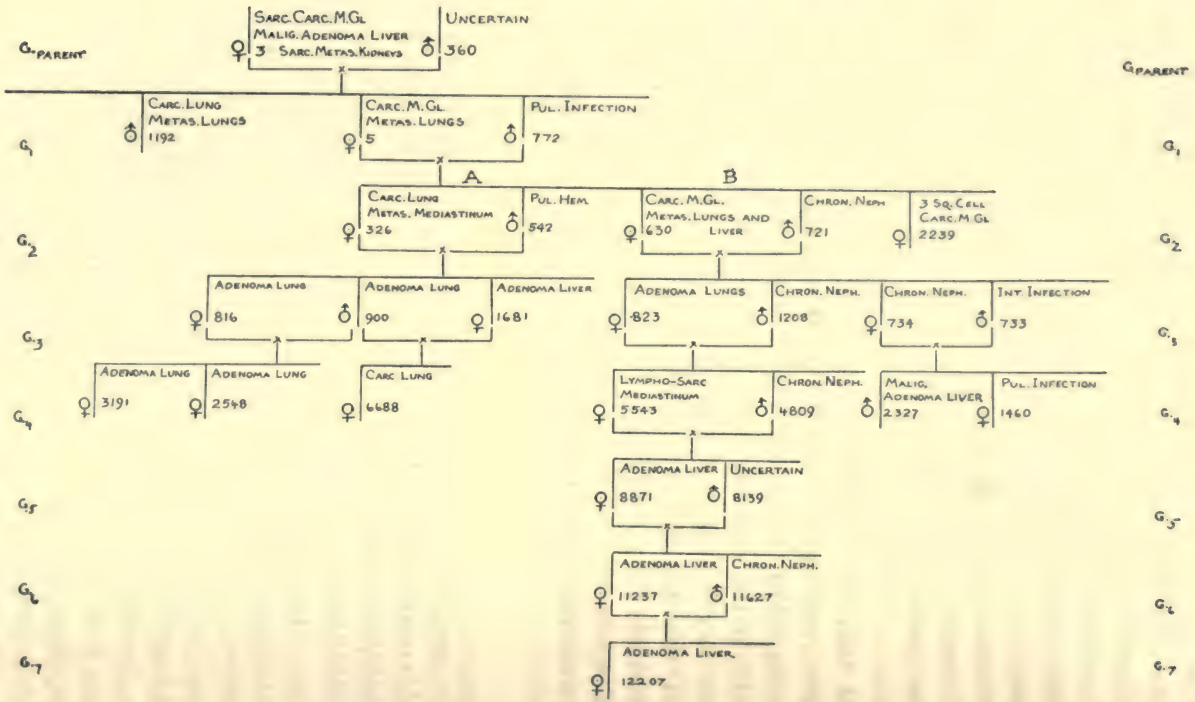


CHART 1

and as secondary neoplasms. In the first hybrid generation, male 1192 had a primary carcinoma of the lung and a secondary carcinoma of the lung. Female 5 had a primary carcinoma of the mammary gland, with multiple secondaries in the lung. Note how the different types and locations of tumors have "segregated out," so that in branch A lung tumors alone have been transmitted, and a 100 per cent lung tumor strain is being extracted. In branch B liver tumors have predominantly been transmitted, from female 630 with a secondary tumor in the liver, so that in one part of the branch a 50 per cent liver tumor strain is being extracted.

In these two branches of the strain, then, female 3 introduced:

a. Primary carcinoma of the mammary gland, in females 5, 2239, and 630.

b. Primary adenoma of the liver, in females 1681, 8871, 11237, and 12207, and in male 2327. (Note the three consecutive generations of adenoma of the liver.)

c. Secondary carcinoma of the liver, in female 630.

d. One case of sarcoma, in female 5543.

Parent male 360 introduced:

a. Primary carcinoma and adenoma of the lung, in males 1192, and 900, and in females 326, 816, 823, 3191, 2548, and 6688.

b. Secondary carcinoma of the lung, in male 1192 and in females 5 and 630.

c. Primary tumor of the mediastinum, in female 5543.

d. Secondary tumor of the mediastinum, in female 326.

Note how female 5543, with a lymphosarcoma of the mediastinum, derived the type of her tumor from her maternal ancestor (female 3) and the location of her tumor from the paternal ancestor, male 360 (who carried these tumors potentially, and transmitted them in every strain into which he was crossed, although he himself did not develop them), just as in the human species, a child might inherit curly red hair from a mother with curly black hair, and a father with straight red hair.

Note how female 5 derived her primary tumor from the maternal ancestor, and her secondary tumor from the paternal ancestor, and how female 630 derived her primary tumor from

the parent female, the secondary in the lungs from the parent male, and the secondary tumor of the liver from the parent female—a marked demonstration of the "segregating out" of these things, which is *the heredity test.* Note how, after the second hybrid generation, no further mammary gland tumors occurred in either of these two branches.

Note that there occur no tumors, either primary or secondary, which were not introduced by the parents. Note that the secondary tumors occur in the same organs as the primary tumors.

Chart 2 shows parts of strains 48 and 292. These are two strains derived from the same original mating as was strain 215, that is, female 3 (with a sarcoma-carcinoma of the mammary gland, a malignant adenoma of the liver, and a metastatic sarcoma of the kidney), and male 360 who introduced carcinoma of the lung and the mediastinum. Here again the particular types and locations of tumors introduced by the parents have "segregated out" and been transmitted to the resulting strains, both as primary and as secondary neoplasms.

In the first hybrid generation female 5 derived from the mother a carcinoma of the mammary gland, and from the father a secondary carcinoma of the lung. In the second hybrid generation, female 37 derived from the parent male a carcinoma of the lung, with a secondary carcinoma in the mediastinum.

In the third and fourth hybrid generations, note the outcropping of sarcoma, both primary and secondary, in females 1454, 26, 348 and 396. Liver tumors derived from female 3, also occur in these strains, as shown in females 5743, and 26, and in male 11836. There is also a "pre-cancerous" liver in female 399. Here also lung tumors, derived from male 360, occur primary in females 37 and 399 and in male 11836; and secondary in females 5, 5743, and 396.

Note the occurrence of secondary *carcinoma* of the mediastinum in female 37, of secondary *sarcoma* of the mediastinum in female 396; of secondary *carcinoma* of the lung in female 5, and of secondary *sarcoma* of the lung in females 5743 and 396. Note the three cases of secondary sarcoma in the spleen in females

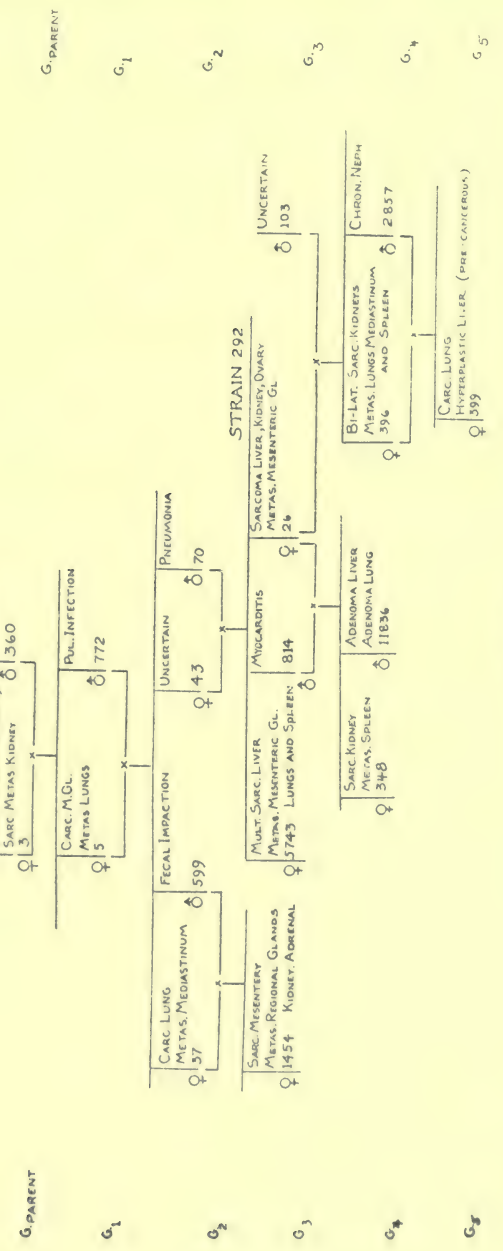

PARTS OF STRAIN 48 AND 292
G. PARENT
G1
G2
G3
G4
G5
SARC. CARC. M.GL. ADENOMA LIVER (MALIG) SARC METAS KIDNEY
♀ 3
UNCERTAIN
♂ 360
CARC. M.GL. METAS LUNGS
♀ 5
PUL. INFECTION
♂ 772
CARC. LUNG METAS. MEDIASTINUM
♀ 57
FECAL IMPACTION
♂ 599
UNCERTAIN
♀ 43
PNEUMONIA
♂ 70
STRAIN 292
SARC. MESENTERY METAS. REGIONAL GLANDS KIDNEY. ADRENAL
♀ 1454
MULT. SARC. LIVER METAS. MESENTERIC GL. LUNGS AND SPLEEN
♀ 5743
MYOCARDITIS
♂ 814
SARCOMA LIVER, KIDNEY, OVARY METAS. MESENTERIC GL
♀ 26
UNCERTAIN
♂ 103
SARC. KIDNEY METAS. SPLEEN
♀ 348
ADENOMA LIVER ADENOMA LUNG
♂ 11836
BI-LAT. SARC. KIDNEYS METAS. LUNGS MEDIASTINUM AND SPLEEN
♀ 396
CHRON. NEPH
♂ 2857
CARC. LUNG HYPERPLASTIC LIVER. (PRE CANCEROUS)
♀ 399

CHART 2

5743, 348, and 396. Rarely in this stock, except in strains derived from female 3, have there been any tumors of the spleen either primary or secondary.

Here again, after the first hybrid generation, mammary gland tumors disappear—another indication of how these things "segregate out." The mammary gland tumors preponderated in other strains derived from the same original mating. The dominating tumors, both primary and secondary, in these strains are in the liver, kidney, spleen, mesentery, lungs, and mediastinum.

In these strains, then, female 3 introduced:

a. Primary carcinoma of the mammary gland, in female 5.

b. Primary adenoma of the liver, in female 399 and in male 11836.

c. Primary sarcoma of the liver, in females 5743 and 26.

d. Primary sarcoma of the kidneys, in females 26, 348, and 396.

e. Secondary sarcoma of the kidneys, in female 1454.

f. Secondary sarcoma of the spleen, in females 5743, 348, and 396.

g. Primary sarcoma of the mesentery, in female 1454.

h. Secondary sarcoma of the mesentery, in females 1454, 5743, and 26.

Parent male 360 introduced:

a. Primary lung tumors, malignant and not yet malignant, in females 37 and 399, and in male 11836.

b. Secondary lung tumors, in females 5743 and 396.

c. Secondary carcinoma of the mediastinum, in female 37.

d. Secondary sarcoma of the mediastinum, in female 396.

Note here again, how, though they are much fewer, the secondary tumors arise in the same organs as do the primary.

Chart 3 shows a part of strain 65, branch 2. The parent female, 5738, had three carcinomas of the mammary gland and metastatic tumors in the lungs. She was mated with her brother, male 782, who died of uncertain causes, but who was proved heterozygous to tumors of the mammary gland (he carried them potentially and transmitted them, but did not himself develop them). Note here that with one exception

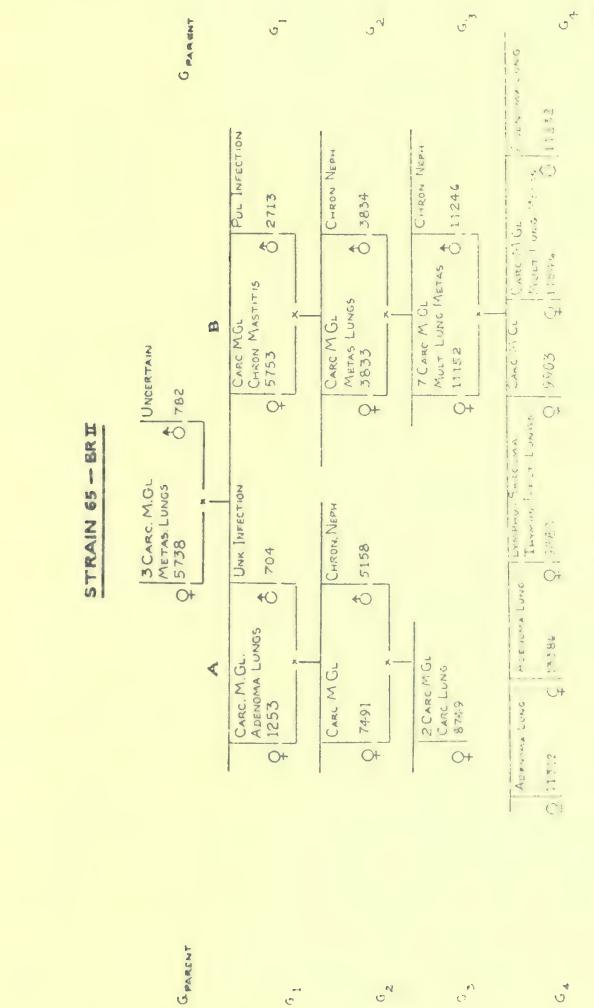

STRAIN 65 — ER II
G PARENT
3 CARC. M.GL. METAS LUNGS 5738
UNCERTAIN 782
A
B
CARC. M.GL. ADENOMA LUNGS 1253
UNK INFECTION 704
CARC M.GL CHRON MASTITIS 5753
PUL INFECTION 2713
CARL M GL 7491
CHRON. NEPH 5158
CARC M.GL METAS LUNGS 3833
CHRON NEPH 3834
2 CARC M.GL CARC LUNG 8749
7 CARC M GL MULT LUNG METAS 11152
CHRON NEPH 11246
G1
G2
G3
G4

CHART 3

(female 9888 who had a lymphosarcoma of the thymus) the only tumors which occurred were carcinoma of the mammary gland and lung tumors, primary and secondary.

Note the five consecutive generations of carcinoma of the mammary gland in branch B, all but females 5753 and 9903 showing lung metastases. Note the heavy percentage of lung tumors primary and secondary in both branches of the strain. Note the negative evidence of the "segregating out" of the different types and locations of tumors, both primary and secondary, in the complete absence from this strain of all tumors of the liver, kidney, mesentery, spleen, pancreas, uterus, etc.

Chart 4 shows a part of strain 338, branch V, A, with partial ancestry. The parent female 3920 had an adenoma of the liver. Note the predominance of primary liver adenoma in this strain, 7 cases (over 43 per cent) and the occurrence of two cases of secondary liver tumor in the same strain, that is, female 8865 with an osteoid sarcoma of the mammary gland, secondary in the liver, and her grandson, male 16370 with an osteosarcoma of the leg, metastasizing in the liver, and also a mesothelioma of the testicle. (Testicle tumors also occur in this strain.)

Chart 5 shows a continuation of the same strain, strain 338, branch V, A (the data being too extensive for a single chart). Chart 5 begins with generation 3 of the family, the mating being between female 8619 and male 8751. Generations 7, 8, and 9 are added to the family in this chart. Note here, also, the further occurrence of liver tumors, there being one additional primary, in female 30469, who showed a primary liver adenoma in addition to seven carcinomas of the mammary gland, a primary lung adenoma, a typical uterine fibroid, and a diffuse endothelial hyperplasia of the glands and spleen. (Needless to say, the mouse was riddled with tumor, little but neoplasm remained.) There were also in this part of the family, two additional cases of secondary liver tumor, *one carcinoma* and *one sarcoma*, that is, female 22263 with three carcinomas of the mammary gland and a sarcoma in the peritoneum near the stomach, with *sarcoma metastases* in the liver, pancreas, and uterine wall, and female 30501 of generation 9, with two carci-

PART OF STRAIN 338 — BR.V A — WITH PART OF ANCESTRY

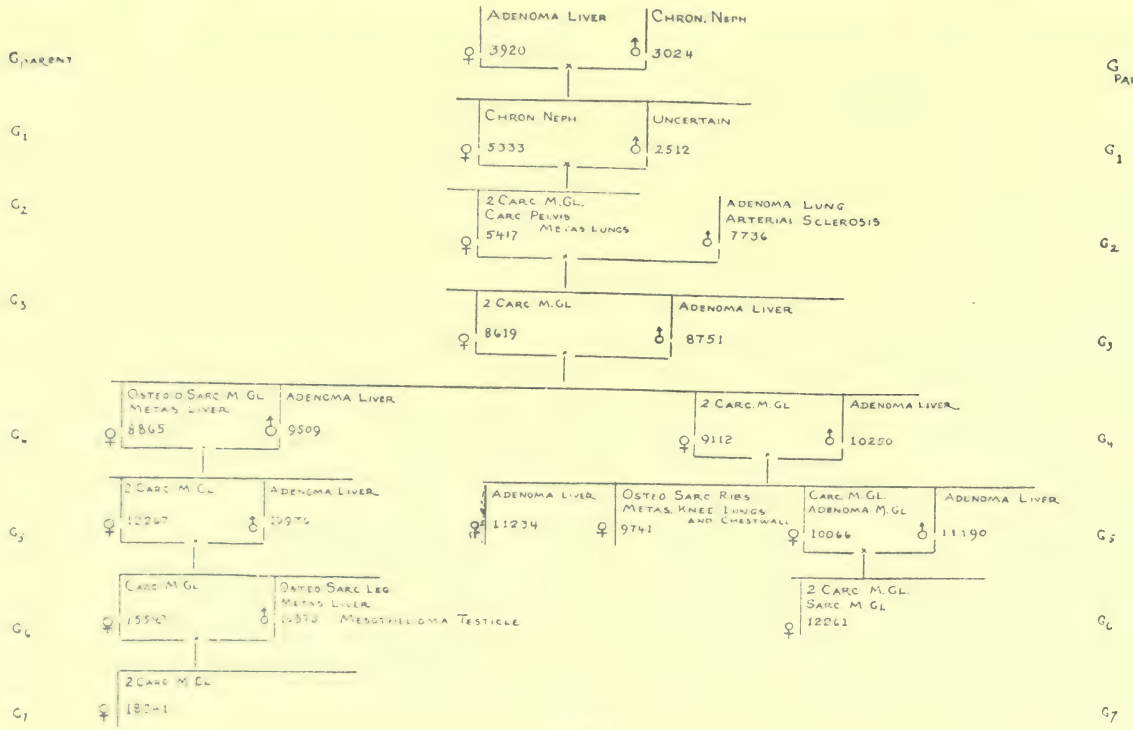


CHART 4

nomas of the mammary gland and the liver absolutely riddled with *carcinoma secondaries*.

It is interesting to note here also the two occurrences of uterine neoplasm, *primary* in female 30469, and *secondary* in female 22263, as this is one of the strains carrying most of the uterine tumors in this stock.

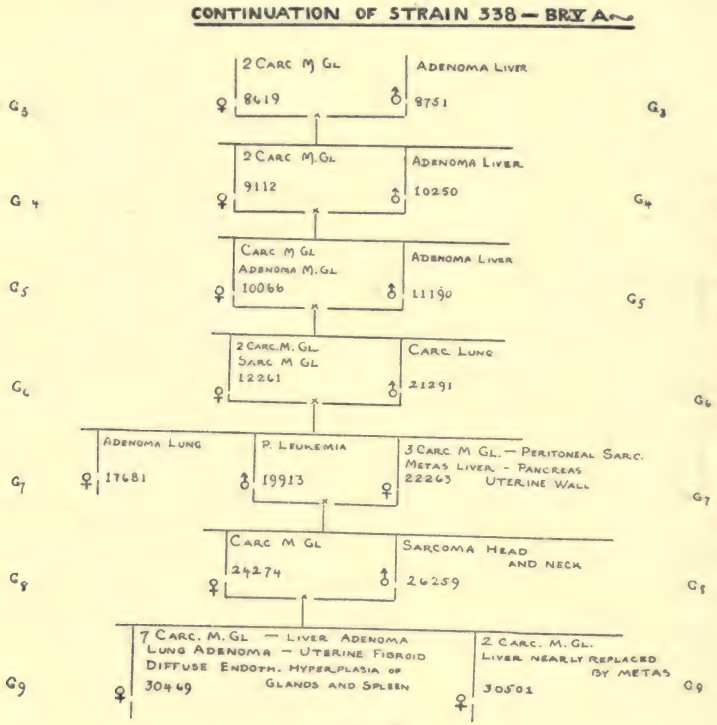


CHART 5

The pancreatic secondary sarcoma also is extremely interesting, as this is the strain which carries the only other pancreatic neoplasms which have occurred in this stock.

The complete ancestry of this strain is shown in charts 6 and 7, chart 6 being the ancestry of parent female 3920, and chart 7 the ancestry of parent male 3024. The prominence of liver tumors, primary and secondary, in the resulting strain is due to the fact that it was bred in from both sides of the family; the

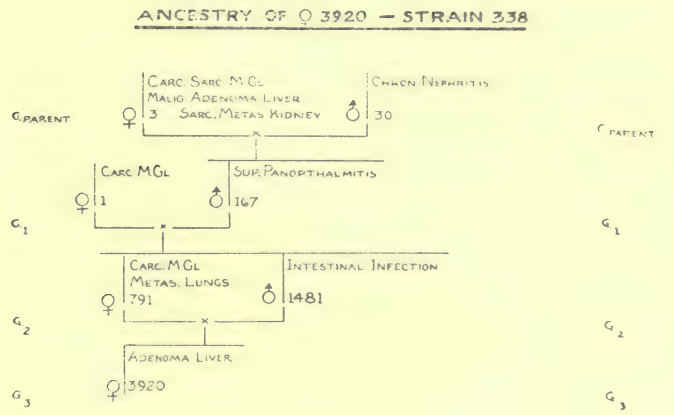


CHART 6

strain thus received "a double dose." Note also the marked outcropping of sarcoma in strain 338, derived from original parent female 3. Sarcoma also was bred in from both sides of the ancestry.

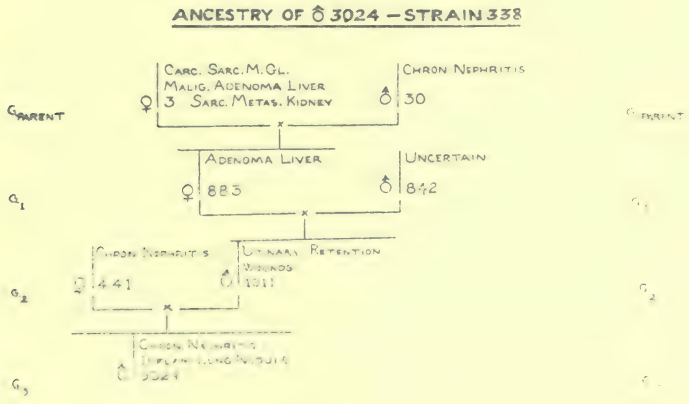


CHART 7

Chart 8 shows a part of strain 465, which was derived from the same original ancestry as strain 338 (ancestry shown in charts 6 and 7). Note here also, the same appearance of liver tumors, two primary and one secondary. This gives us, then,

in these two small strains (338, branch V, A and 465) derived from identical original ancestry, 10 primary and 5 secondary liver tumors. Including the ancestry, the number is 13 primary and 5 secondary liver tumors. This is a very high occurrence of liver tumors, both primary and secondary, especially when one considers the fact that outside of this stock there is just one mouse liver tumor on record, a primary tumor of the liver, re-

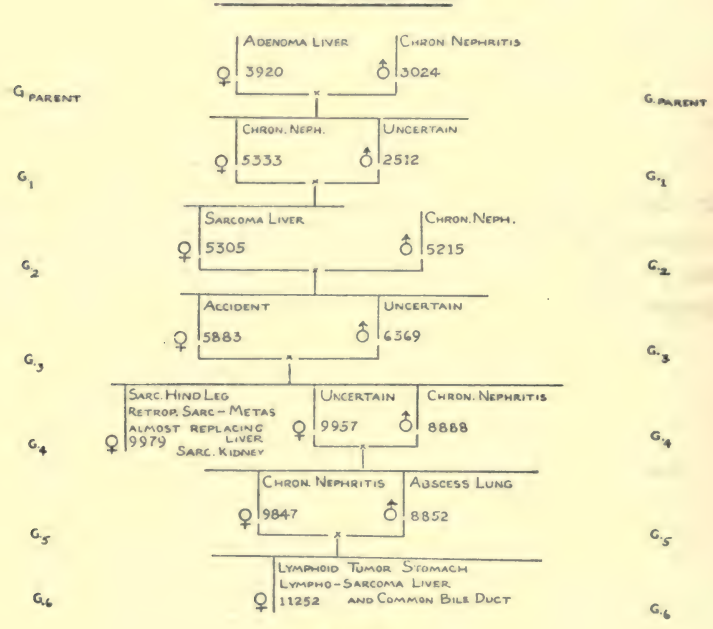


CHART 8

ported by the Imperial Cancer Research Fund of England. This liver tumor occurrence (in the Slye stock) within a single strain, makes a piece of evidence which is conclusive.

These five strains, represented in charts 1 to 8 inclusive (which, it must be remembered, are perfectly typical) furnish both *positive* and *negative* evidence of the "segregating out" of different types and locations of tumors, and their transmission as such, whether as primary or as secondary neoplasms. They

furnish, therefore, both positive and negative evidence that *in any given strain the metastatic tumors (where there are any) tend to occur in exactly the same organs in which the primary tumors of that strain occur.* Note, therefore, that:

1. Every secondary tumor of the liver in this stock (with the possible exception of two osteosarcomas, which metastasized into nearly every organ in the body, in two mice whose strains have not yet been completely analyzed) has occurred in strains carrying primary liver tumors.

2. Every secondary tumor of the kidney in this stock has occurred in strains carrying primary kidney tumors.

3. Every secondary lung tumor in this stock has occurred in strains carrying primary lung tumors (with the possible exception of the one lung metastasis in strain 164, branch IV, line C).

4. Every secondary tumor of the mediastinum in this stock has occurred in strains yielding primary tumors of the mediastinum.

5. Every secondary tumor of the pancreas in this stock has occurred in the strain which yields primary pancreatic tumors.

6. Every secondary tumor of the spleen in this stock, has occurred in strains carrying primary spleen tumors.

7. Every secondary uterine tumor in this stock has occurred in strains carrying primary uterine tumors.

When this stock, or any other, has been completely analyzed, there is little doubt that it will show that every secondary tumor of any organ whatever has arisen in a member of a strain which has shown, or will show, some cases of primary tumors in that organ (however few they may be). There is little doubt that strain 164, branch IV, line C, of this stock will in time yield a primary lung tumor, although it has not done so yet. There is a very slight inheritance of lung tumor neoplasm tendency in this line of the strain, and it is therefore to be expected that lung tumors of any kind will occur but seldom.

The complete analysis of any strain is a very long and difficult process, and the apparent testimony of the frequent occurrence of secondaries in man in tissues where primary tumors rarely occur is of no help here, as no human strain has ever been even partially analyzed.

Female 3 is listed in these charts as having a sarcoma-carcinoma of the mammary gland. This tumor was unquestionable carcinoma in some parts and apparently sarcoma in other parts. There has been considerable difference of opinion concerning these mixed tumors. Slye, Holmes, and Wells (5) Loeb (6), Lewin (7), Bashford (8), Woglom (9), and some others, diagnose them as such, while Le Count (10) and Ewing (11), etc., consider them to be entirely carcinoma, with pressure distortion of the cells simulating sarcoma in some areas.

The biological evidence of the work in this laboratory unquestionably supports the opinion that there are these mixed tumors. Female 3 with such a tumor, has unquestionably transmitted carcinoma in every strain into which she has been hybridized; and she has equally certainly transmitted sarcoma in some branch of every strain into which she has been crossed (see charts 1, 2, 4, 5 [female 3 shown in ancestry charts 6 and 7], 8 (same parentage charts 6 and 7), Chart 9).

I have preferred the term sarcoma-carcinoma to represent this type of tumor, because according to the biological evidence, it is not a sarcomatous carcinoma, nor a carcinomatous sarcoma, but rather a sarcoma plus a carcinoma, each type of tumor being transmitted separately, as such. Biological evidence, as manifested in heredity behavior, is too fundamental to be ignored.

POINT II

In certain strains there is a tendency for primary tumors to metastasize in certain organs; whereas, in other strains, tumors of the same type, primary in the same organ, even when they are of older and of larger growth, fail to metastasize into those organs.

Chart 9 shows part of strain 73, which was derived from the same female 3, mated this time with male 30. Male 30 came from a strain carrying tumors of the lung, mediastinum, and diaphragm, and was proved heterozygous to tumors of these organs, having been tested in various crosses.

The son of this mating, Jap. W. F. male, died before autopsies were made, and consequently the cause of his death is not known.

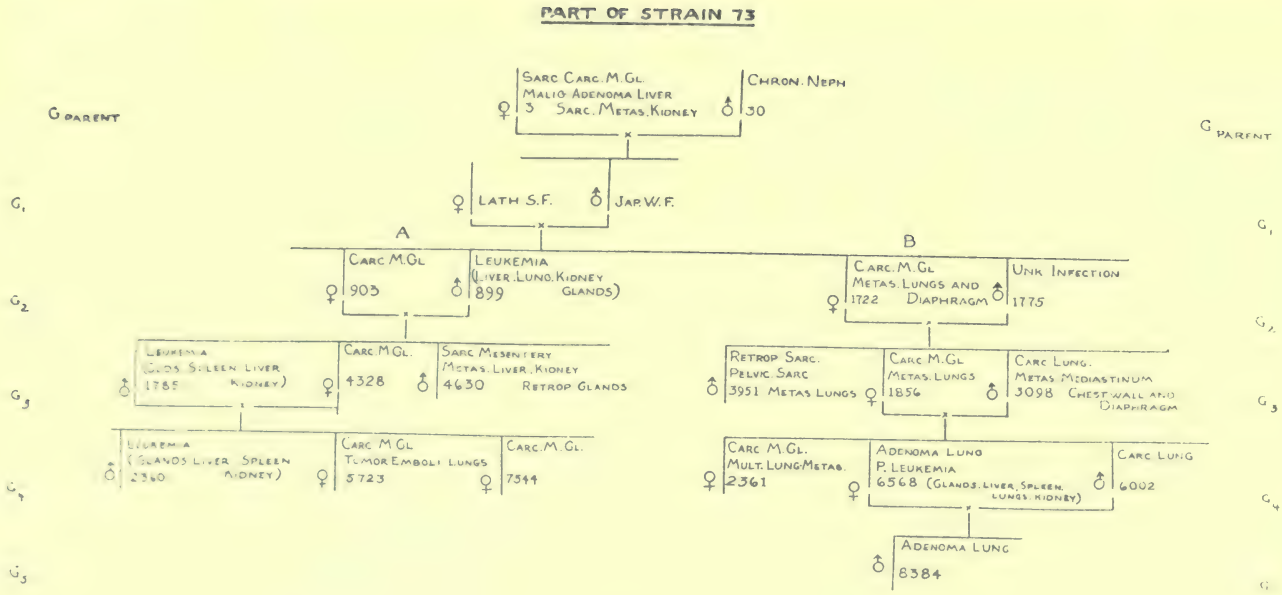

PART OF STRAIN 73
G PARENT
SARC. CARC. M. GL. MALIG. ADENOMA LIVER SARC. METAS. KIDNEY 3
CHRON. NEPH 30
LATH. S.F.
JAP. W.F.
A
B
CARC. M. GL. 903
LEUKEMIA (LIVER. LUNG. KIDNEY GLANDS) 899
CARC. M. GL. METAS. LUNGS AND DIAPHRAGM 1722
UNK. INFECTION 1775
LEUKEMIA (GLDS. SPLEEN LIVER KIDNEY) 1785
CARC. M. GL. 4328
SARC. MESENTERY METAS. LIVER. KIDNEY 4630 RETROP. GLANDS
RETROP. SARC. PELVIC. SARC 3951 METAS. LUNGS
CARC. M. GL. METAS. LUNGS 1856
CARC. LUNG. METAS. MEDIASTINUM 3098 CHEST WALL AND DIAPHRAGM
LEUKEMIA (GLANDS LIVER SPLEEN KIDNEY) 2360
CARC. M. GL. TUMOR EMBOLI LUNGS 5723
CARC. M. GL. 7544
CARC. M. GL. MULT. LUNG METAS. 2361
ADENOMA LUNG P. LEUKEMIA 6568 (GLANDS. LIVER. SPLEEN. LUNGS. KIDNEY)
CARC. LUNG 6002
ADENOMA LUNG 8384

CHART 9

He was crossed with a Lathrop silver-faun female, who also died before autopsies were made. Their offspring, however, are shown in two branches, A and B.

Note how in branch B, every mammary gland carcinoma metastasized into the lungs; even the retroperitoneal and pelvic sarcomas (male 3951) metastasized in the lungs. Note also that with the exception of male 1775 this branch shows 100 per cent of lung tumors, primary and secondary. Note the two generations of metastasis into the diaphragm, female 1722 and male 3098.

On the other hand, in branch A, carcinomas of the same type and of older and larger growth, failed to metastasize into the lungs (females 4328, 5723, and 7544). Note that in female 5723, although the mechanical basis for lung secondaries was present in multiple tumor emboli throughout the lungs, no lung tumor developed. Note that the sarcoma of the mesentery in male 4630 metastasized into the liver, kidney, and retroperitoneal glands, but not into the lungs.

Note the three consecutive generations of leukemia in this strain, in males 899, 1785, and 2360; and the further fact that, although the lungs are one of the principal organs for leukemic infiltration, in males 1785 and 2360 there was no infiltration in the lungs, while in male 899 the lung infiltration was very slight. On the other hand, the marked leukemic infiltration in this family was in the same organs in which the tumors of the parent female occurred, that is, the liver and kidney. The metastatic tumors, also, in this family were in these same organs.

In branch B, however, the 100 per cent lung tumor strain, female 6568 who had pseudoleukemia along with an adenoma of the lung, showed marked infiltration of pseudoleukemia throughout the lungs.

We have here, in these two branches of the same strain, a striking illustration of point II: In branch B all tumors metastasize into the lungs, and pseudoleukemia picks out the lungs predominantly; while in branch A, the tumors of the same type in the same organ and of older and larger growth fail, in every case, to metastasize into the lungs. Even the leukemic infiltration fails to take hold of the lungs in this branch.

Chart 10 shows part of strain 405. In this branch of the family, after the parent generation, although there are five straight generations of mammary gland carcinoma, none of these tumors metastasized into the lungs. Note also the complete absence of primary lung tumors. This branch of the family

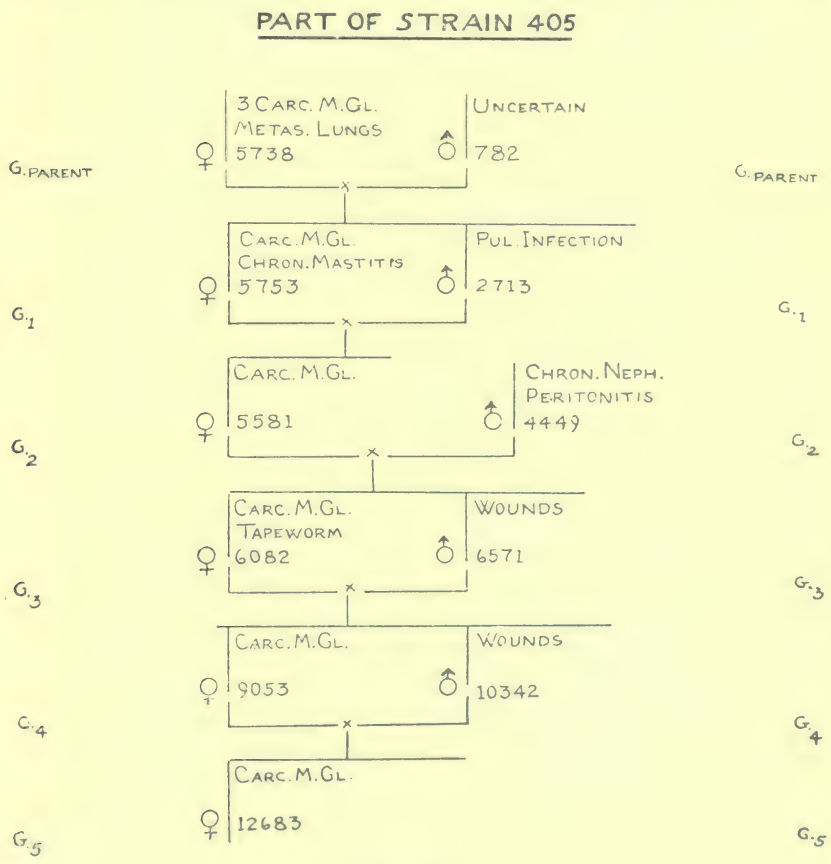


CHART 10

did not inherit lung tumors, and none occurred, either primary or secondary.

Chart 11 shows one branch of strain 508. Here again through five consecutive generations of mammary gland carcinoma, no

lung metastasis occurred. Even where the presence of multiple tumor emboli in the lungs gives certain evidence that cells from the primary tumor were carried to the lungs (in females 7821 and 9260) no lung tumor occurs. The mechanical basis was there; the tumor growth did not follow. This strain also shows no primary lung tumors.

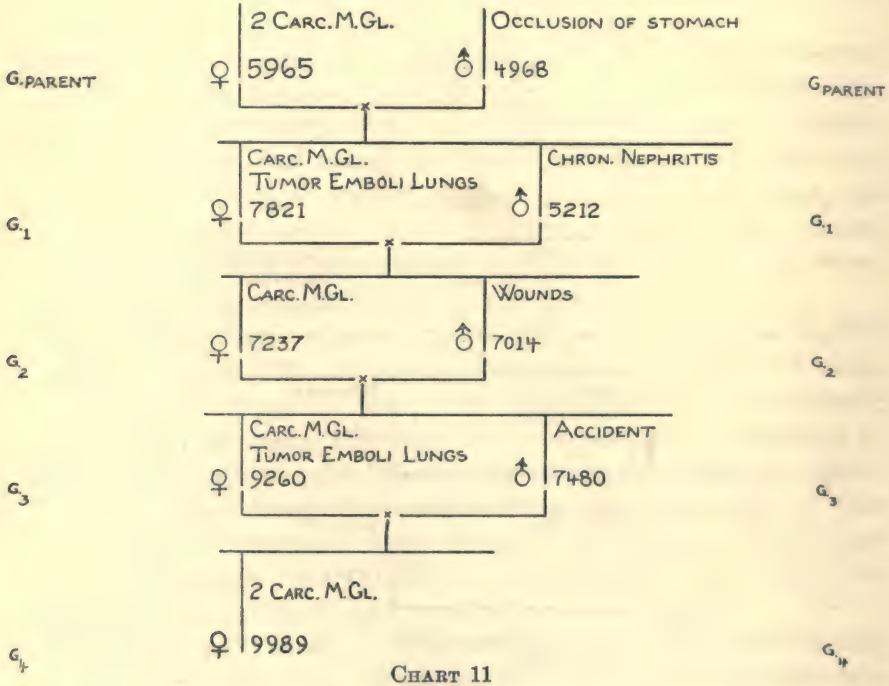


CHART 11

Chart 12 gives a summary of strain 164, branch IV, families A, B, and C. It is shown in summary only in order to get the data within a single chart. These branches of the family showed 48 primary tumors, all but one of them highly malignant, with only one case of lung metastasis, in female 10574, a member of line C. Note the marked absence of lung tumors primary and secondary, although lung tumors are second in frequency of all mouse tumors.

The strain also showed 13 cases of pseudoleukemia and one of leukemia. Here again, it is most interesting to note that the six cases of pseudoleukemia which occurred in line B showed not the slightest infiltration in the lungs. The malignant thymus

STRAIN 164 – BR.XX-A-B-C

PRODUCED THE FOLLOWING CASES OF TUMOR

WITHOUT METASTASIS		WITH METASTASIS
CARCINOMA M.GL.	34	1 IN LUNG
SARCOMA M.GL.	2	0
OVARIAN ADENOMA	6	0
MALIG. THYMUS LYMPHOMA DID NOT INFILTRATE LUNGS	2	0
LYMPHO-SARCOMA THYMUS DID NOT INFILTRATE LUNGS	1	0
SQ.CELL CARC. BACK	1	0
SP. CELL SARC. BACK	1	0
SQ.CELL CARC FACE	1	0
SP CELL SARC.FACE	1	0
RETROPERITONEAL SARCOMA	1	0
TOTAL	48	1
PSEUDO LEUKEMIA	14	

NOTE THE ABSENCE OF PRIMARY LUNG TUMOR AND THE SINGLE CASE OF LUNG METASTASIS

CHART 12

tumors in this line of the family also failed to infiltrate the lungs, although they went extensively into the chest wall and mediastinal glands. The seven cases of pseudoleukemia which fell in line C of this strain, showed a very slight infiltration of the lung. This is the same line which showed one case of lung metastasis.

We have then, in this strain, lines A and B, a marked illustration of the failure of the lungs to yield at all to tumor, primary or secondary. In other words, the lungs of this family do not show neoplastic proliferation, nor do they accept neoplastic secondaries or leukemic growth in spite of the certain and marked presence of the ordinary provocation for such proliferation. In line C of the strain, a slight tendency of the lung to proliferation is present.

This strain was derived by the hybridization of an absolutely nontumorous strain of house mice, in my hands many years, strain 358, with albino strain 146, which carried many tumors, and furnished some 100 per cent tumor families. This strain gives another marked illustration of the segregating out of these things; the tendency to neoplastic lung proliferation, either primary or secondary, failed to be transmitted at all to lines A and B of branch IV of the strain, and only slightly to line C. Note the striking contrast between this strain with no lung tumors, and strain 522 (chart 15) showing 100 per cent lung tumors (p. 167).

These four strains represented in charts 9 to 12 inclusive (which are perfectly typical) furnish both positive and negative evidence of the segregating out of different types and locations of tumors, and their transmission as such, whether as primary or as secondary neoplasms.

They furnish therefore evidence that *in certain strains there is a tendency for tumors to metastasize into certain organs, whereas in other strains, tumors of the same type in the same organ, even when they are of older and of larger growth, fail to metastasize into those organs.* Moreover in these strains, leukemia and pseudoleukemia tend to infiltrate the same organs which show primary and secondary neoplasms, and fail to take hold of the same organs which do not yield to primary or secondary tumors.

In this connection, it is interesting to analyze the results of various experiments to test the effect upon metastasis formation of making exploratory incisions into tumors, of massaging tumors, etc. Let me indicate what would be a complete biological control in such experiments.

TWO ILLUSTRATIONS OF COMPLETE BIOLOGICAL CONTROL.

I

Strain 164 branch C of the Slye stock, during its twelve years of existence has consistently yielded 0.01 per cent of lung tumors primary and secondary, leukemic and pseudoleukemic infiltration of the lung, and infiltration into the lungs from malignant thymus tumors.

If now we use tumorous mice from strain 164 branch C, and excise for diagnosis pieces of their tumors or massage their tumors or otherwise manipulate them, and if we find at autopsy that the group of mice whose tumors have been manipulated show little or no higher percentage than 0.01 per cent of lung tumor metastasis, and that the group which is not manipulated runs true to this 0.01 per cent lung tumor metastasis, we shall have a perfect control, and shall be justified in saying, without qualification, that excising a piece of tumor for diagnosis or massaging the tumor, or otherwise manipulating it, had no effect upon the production of lung tumor metastasis.

II

Strain 65 branch II B has during its twelve years of existence produced 35 per cent of lung tumor metastasis.

If now we repeat these same experiments with this strain, and find at autopsy that the group whose tumors have had a piece excised for diagnosis or which have been in any other way manipulated, show little or no increase in lung tumor metastasis over 35 per cent, and if the unmanipulated group runs true to this percentage of lung tumor metastasis, we shall have a perfect biological control of the experiments, and shall be justified in saying without qualification that the excision of a piece of tumor for diagnosis or any other form of manipulation had no effect upon the production of lung tumor metastasis.

THREE ILLUSTRATIONS OF THESE EXPERIMENTS WITH INADEQUATE BIOLOGICAL CONTROL

I

Strain 164 branches A and B have during their existence for twelve years, without exception of any sort, yielded 0 per cent of lung tumors primary or secondary even where tumor emboli were present in the lungs, 0 per cent of leukemic or pseudoleukemic infiltration into the lungs, and 0 per cent of infiltration into the lungs by extension from thymus, diaphragmatic, or other adjacent malignant tumors.

If now we repeat these same experiments with these branches of the strain, we shall find at autopsy 0 per cent of lung tumor metastasis in every group of mice, whether or not we excise a piece of tumor, or massage the tumor, or otherwise manipulate it. That is, a strain whose lungs have been proved not to yield to tumor emboli is not a strain fitted to show the effect of these or any other types of manipulation.

II

Strains 139, 522, 73, branch B, etc., have during their many years of existence without exception furnished 100 per cent of lung tumors, primary and secondary, infiltration into the lungs by leukemia and pseudoleukemia, and extension infiltration into the lungs from malignant thymus and other adjacent tumors.

If now we repeat these same experiments with these or any other 100 per cent lung tumor strains, we shall find at autopsy an exceedingly high percentage of lung tumor metastasis, probably 100 per cent in every group whether excised or not. That is, a strain which for twelve years has shown 100 per cent of lung tumor is not a strain fitted to show the effect upon lung metastasis of this or any other type of manipulation.

III

Groups of mice even when purchased from one dealer are mixed lots, secured from many divergent sources, and wholly unanalyzed

as to tumor potentiality. They may include some individuals from 0 per cent lung tumor strains, some from 100 per cent, some from 35 per cent, etc. If we take such groups of mice and repeat these same experiments, we shall have *no biological control*, and we shall not be justified in saying whether the lung tumor metastases found at autopsy were or were not caused by the excision of a piece of tumor for diagnosis, or by massage, or whatever form of artificial manipulation may have been used.

It is greatly to be deplored that the vast majority of experimental studies in animal pathology and bacteriology have not been and are not as yet being conducted with animals whose hereditary potentialities have been studied. It is being taken for granted that pathological conditions found in the animal after experimental procedure, are the result of such experimental procedure. No allowance is being made for heredity.

Until stocks of animals to be used in such experiments, have been thoroughly tested out as to their inherited potentialities, such experiments are lacking in adequate control, as the factor of heredity is not being considered, although it is tremendously potent. Heredity alone would be sufficient to give the exact results attained in countless experiments which have been conducted without any effort to study or control the heredity factor. Moreover, most of these studies in metatasis production by the artificial manipulation of the primary tumor have been conducted with grafted tumors. As has frequently been stated in the series of cancer studies from this laboratory, the biological difference between spontaneous and grafted tumors is so fundamental and so complete, that the behavior of grafted tumors has practically no bearing upon the behavior of spontaneous tumors.

POINT III

Individuals with secondary tumors in any given organ seem to be as potent as individuals with primary tumors in the same organ to transmit by heredity primary tumors in that organ.

Note chart 13, showing part of strain 304 with ancestry. Here two successive generations of females (529 and 467) with second-

PART OF STRAIN 304 WITH ANCESTRY

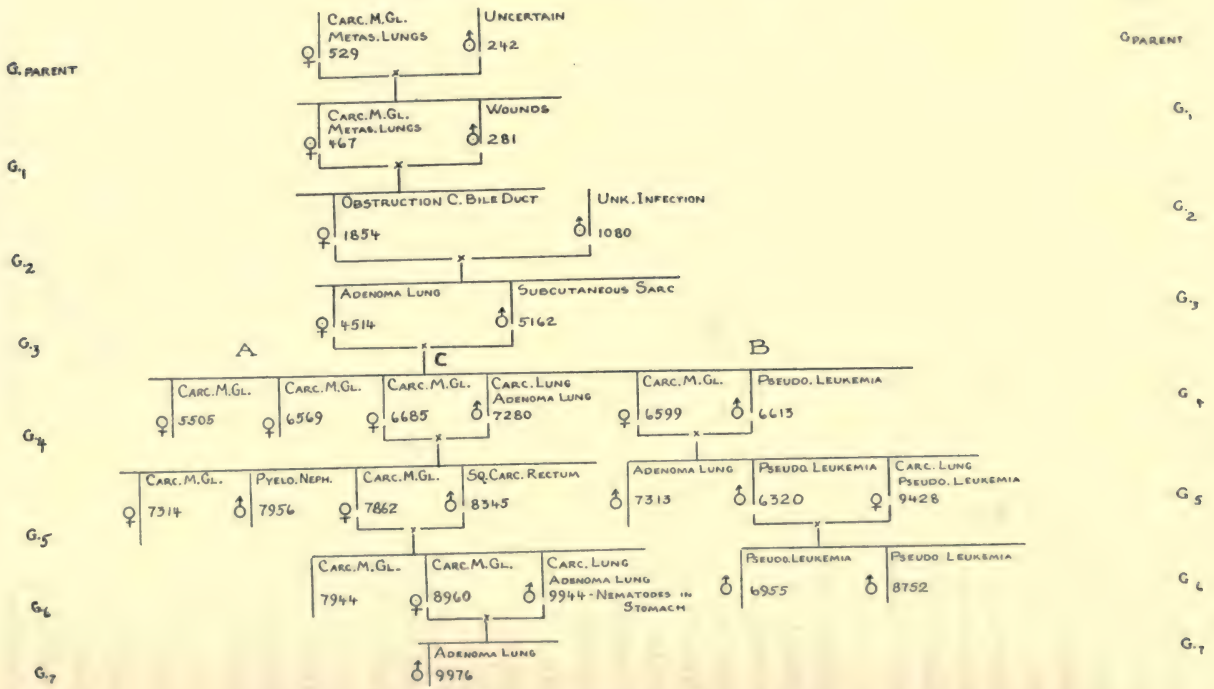


CHART 13

ary lung tumors, have originated a strain which, in line C, is producing a 44.4 per cent primary lung tumor strain. The combination of both lines B and C shows 33.3 per cent of primary lung tumors. Note the 100 per cent pseudoleukemia family being extracted in branch B, in all of which the lungs were infiltrated extensively.

Again, in chart 14, strain 338 branch VI, a 100 per cent lung tumor strain is being extracted from the original mating of female

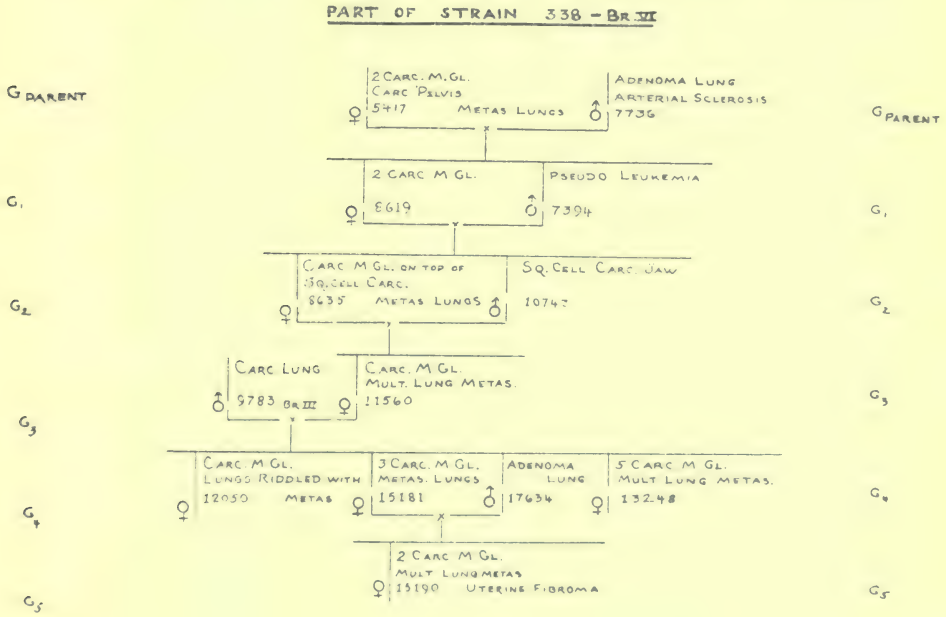


CHART 14

5417, with a *secondary* lung tumor, with male 7736, with a primary lung tumor; that is, the metastatic lung tumor is behaving like a primary lung tumor in helping to produce a 100 per cent lung tumor strain, primary and secondary.

Refer again to chart 1 (page 142), showing part of strain 215, and note how female 630 (parent of branch B) with a metastatic carcinoma of the liver, produced three straight generations of primary liver adenoma (not yet malignant) females 8871, 11237,

and 12207, as well as a primary malignant adenoma of the liver in male 2327. Note also how her secondary lung tumor was followed by a primary lung tumor in female 823 (G_3).

Refer again to chart 2 (page 145), showing strains 48 and 292, and note how the secondary sarcoma of the kidney in parent female 3, resulted in primary sarcomas of the kidneys in females 26, 348, and 396. Refer again also to chart 3 (page 147), strain 65, and note how secondary lung tumor in the parent female 5738, introduced primary lung tumors in the strain, in females 1253, 8749, 11512, and 13386, and in male 11832. Note that the thymus sarcoma in female 9888 was markedly infiltrating the lungs, while in strain 164 Br. IV, lines A and B, which did not produce lung tumors of any sort, either primary or secondary and in which leukemia and pseudoleukemia failed to infiltrate the lungs, the thymus tumors also failed to infiltrate the lungs.

Note also how in chart 15, strain 522, female 5636, with a secondary lung tumor (G_2), mated with male 8102 with a primary lung tumor, produced a 100 per cent primary lung tumor strain (center of the chart). The pseudoleukemia in this 100 per cent lung tumor strain, viz., female 14033, showed marked infiltration in the lungs.

From these charts, viz., 13 to 15 inclusive, and also from charts 1, 2, and 3 (which are perfectly typical) it is evident *that individuals with secondary tumors in any given organ seem to be as potent as individuals with primary tumors in that organ, to transmit by heredity, primary tumors in that organ.*

DISCUSSION

We have here, in the charts presented in this report, biological evidence of the nature of cancer heredity. It is well to bear in mind that until the facts of pathology have been based upon the more fundamental biological facts, it will never be possible to get a complete explanation of the pathological behavior of tissues.

Let me repeat at this point, what I already have stated frequently in previous publications: the materials used for this study are individuals analyzed as to their hereditary poten-

PART OF STRAIN 522 WITH PARTIAL ANCESTRY

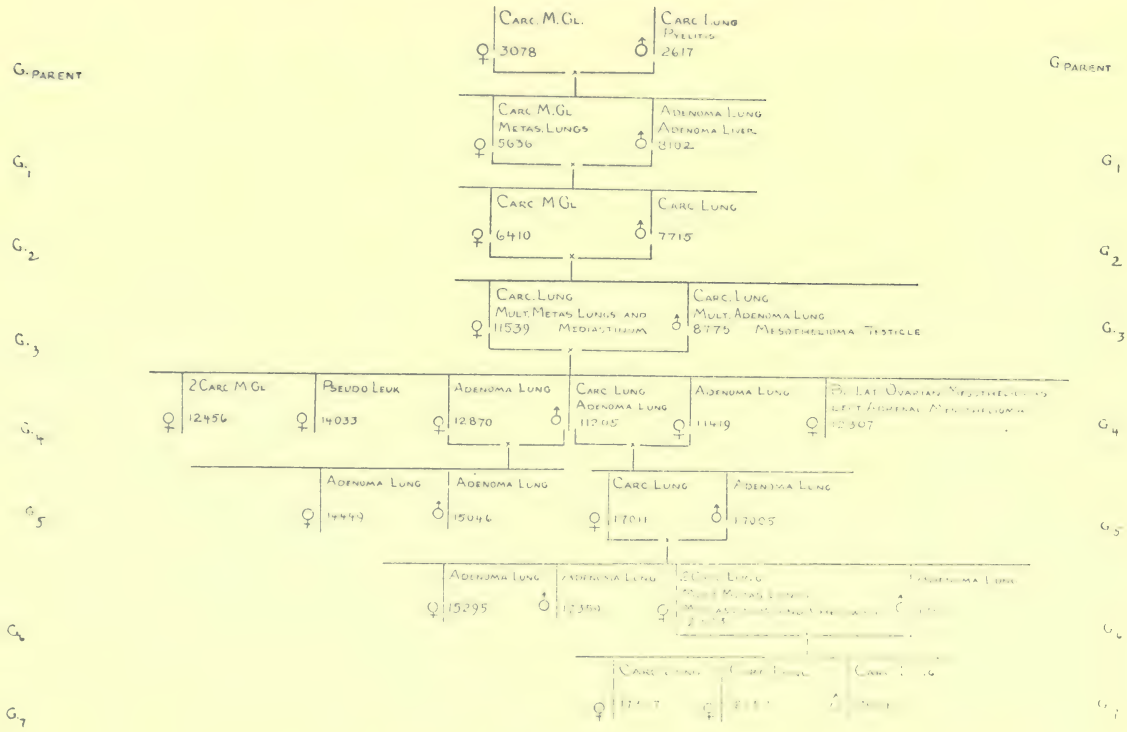


CHART 15

tialities by every possible test. The tumors are all spontaneous, arising without interference of any sort except that of selective breeding. By the process of selective breeding alone, using analyzed individuals as material, it is possible to extract strains in which the lungs do not react with either primary or secondary neoplastic proliferation or leukemic infiltration; or strains in which the liver does not so react, or the kidneys, or the mammary gland tissue, etc.

Etiological meaning. The fact that both primary and secondary tumors of a given organ or organs tend to occur in the same strains, and fail to occur at all in other tumor strains, indicates beyond question that *heredity is a strong factor in determining not only where the primary tumors shall occur, but also where the secondary tumors shall occur.*

The consequent fact, that it is possible to extract strains where only certain organs shall furnish both the primary and the secondary neoplasms, and shall yield to leukemia and pseudoleukemia; and to extract other strains in which these organs never furnish either primary or secondary neoplasms nor yield to leukemic or pseudoleukemic invasion, and that secondary tumors are just as potent as primary tumors in the transmission of primary tumors in any given organ, shows beyond a doubt that the thing which is transmitted in the heredity of cancer is the tendency of a given organ or organs to yield to cancer. This inherited tendency of an organ or organs to yield to cancer is manifested, whether the lesion is primary in that organ, or whether cells from the primary growth lodge in that organ and form a secondary lesion.

The negative evidence on this point is just as conclusive, that is, that in strains from which tumors of a certain organ or organs have been eliminated by heredity, the cells from the primary neoplasm fail to take hold, even when they lodge in such organ, and do not form a secondary growth; and even when a tumor completely surrounds an organ, it fails to penetrate it by extension.

Now what do these things mean? They mean that the tendency to carcinoma segregates out and is transmitted as such;

that the tendency to sarcoma or adenoma, etc., segregates out and is transmitted as such; that a strong tendency to the location of one or more of these types of tumor in a specific organ, such as the liver, the lung, the kidneys, the mammary glands, etc., is transmitted, owing to the segregating out of a peculiar type of tissue in these organs, which will respond in a neoplastic manner to lesions of any kind which furnish a chronic irritation of not too destructive a type. That is, the *tissues of these organs are of the same nature as the ancestral organs from which they are derived. There is a specificity of tissue type from liver to liver, or from kidney to kidney, etc., which will make those organs react in a given way to a given type of irritation.*

Now with each of these things being transmitted as such, it is possible for a parent female with a sarcoma-carcinoma of the mammary gland, a malignant adenoma of the liver, and a secondary sarcoma of the kidney (like female 3) to transmit to the strains derived from her, a tendency to:

1. Carcinoma of the mammary gland.
2. Sarcoma of the mammary gland.
3. Adenoma of the mammary gland.
4. Carcinoma of the liver.
5. Sarcoma of the liver.
6. Adenoma of the liver.
7. Carcinoma of the kidney.
8. Sarcoma of the kidney.
9. Adenoma of the kidney, or any combination of these nine.

If she is mated with a male, either himself having a lung and a mediastinal tumor, or being heterozygous to these locations of tumor (as in the case of male 360), the resulting strains will show:

1. Carcinoma of the mammary gland.
2. Sarcoma of the mammary gland.
3. Adenoma of the mammary gland.
4. Carcinoma of the liver.
5. Sarcoma of the liver.
6. Adenoma of the liver.
7. Carcinoma of the kidney.
8. Sarcoma of the kidney.

9. Adenoma of the kidney.
10. Carcinoma of the lung.
11. Sarcoma of the lung.
12. Adenoma of the lung.
13. Carcinoma of the mediastinum.
14. Sarcoma of the mediastinum.
15. Adenoma of the mediastinum, or any combination of these tumors.

The neoplastic growth may be primary in any one or more of these organs, according to where there is occasion for the primary lesion to occur, and there may be secondary growths in any one or more of these organs, according to where the secondary lesions occur.

Now note that this is exactly what we get from the matings of female 3 (with a sarcoma-carcinoma of the mammary gland, a malignant adenoma of the liver, and sarcoma metastasis in the kidney) with male 360, proved heterozygous to lung and mediastinal tumors. Note especially charts 1 and 2 where the strains were derived from the same original mating of female 3 with male 360; and charts 4, 5, 6, 7, 8, and 9, showing ancestry and strains derived from the mating of female 3 with male 30.

It should not be a matter for surprise that there is an inheritance of a specific type of liver tissue, or kidney tissue, etc., which will tend to respond in a given way to a given irritation or a given lesion. Similar facts of inheritance, such as a tendency to grow tall, with the proper degree of nourishment, etc., are easily accepted. That also is the inheritance of tissues which tend to respond in a given way to a given stimulus. There is here also a certain specificity, as, for example, the tendency to longness in the trunk and shortness of the limbs; or of shortness of the trunk and longness in the limbs, so that a given figure tends to predominate in a given family, just as given types and locations of neoplasms tend to predominate in a given family. Again, it is an accepted expectation that there should be a given nose shape, or shape of lips, or contour of head, inherited within a family; i.e., a specificity of nose tissue, etc., which will insure its growing to a given shape, etc.

This specificity of organ tissue, which shall insure its reacting in a given neoplastic way, to a given chronic irritation, is exactly what we find to be the nature of cancer heredity; and it is obvious that it will make no difference in the nature of the reaction of such specific organ tissue, whether the lesion is primary or secondary.

Heredity, therefore, of a specific type of organ tissue, is here shown to be the fundamental influence in determining the incidence and location of metastatic neoplasms, as well as those of primary neoplasms.

In regard to the relative infrequency of secondary tumors in this stock, the biological evidence is as follows: Many of these strains have been made by hybridizing a tumorous individual with a proved non-tumerous individual. This, of course, is done to test for the Mendelian behavior of cancer in heredity. Now, with the tendency to carcinoma, sarcoma, etc., segregating out, and a specific type of organ tissue segregating out and being transmitted as such, it is evident that any given mouse may inherit a tendency to only one type of tumor, and in only one organ. Other organs, therefore, in such a mouse, refuse to yield to cancer, even where emboli are present in such organs. Many such cases have been pointed out as shown in the charts in this report.

Individuals into whose ancestry (as is the case in much human heredity) an indiscriminate amount of cancer had been bred would show a correspondingly higher percentage of metastasis.

SUMMARY

These studies in the metastasis behavior of spontaneous tumors demonstrate the following facts:

1. In any given strain, the metastatic tumors (where there are any) tend to occur in exactly the same organs in which the primary tumors of that strain occur.

2. In certain strains, there is a tendency for tumors to metastasize in certain organs; whereas in other strains, tumors of the same type in the same organ, even where they are of older and of larger growth, fail to metastasize into those organs.

3. Leukemia and pseudoleukemia, occurring in tumor strains, pick out predominantly the same organs for infiltration which show the primary and secondary tumors of that strain.

4. Tumors do not even invade by extension the organs from which primary and secondary neoplasms have been eliminted by heredity.

5. Individuals with secondary tumors in any given organ, seem to be as potent as individuals with primary tumors in the same organ, to transmit by heredity, primary tumors in that organ.

Therefore:

6. Heredity is a strong factor in determining not only where the primary tumors of a strain shall occur, but also where the secondary tumors shall occur.

7. Heredity is a strong factor in determining what organs of a strain shall yield to the invasion of leukemia and pseudoleukemia.

8. The thing which is transmitted in the heredity of cancer is the tendency of an organ or organs to yield to cancer. This tendency is manifested whether the lesion is primary in that organ, or whether cells from the primary growth lodge in that organ and form a secondary lesion.

9. The tendency to sarcoma, carcinoma, adenoma, etc., segregates out and is transmitted as such.

10. A strong tendency to the location of one or more types of tumor in a specific organ or organs, such as the liver, kidney, pancreas, mammary gland, etc., is transmitted, owing to the segregating out of a peculiar type of tissue in these organs, which will respond in a neoplastic or leukemic manner to lesions of any kind which furnish a chronic irritation of not too destructive a type.

11. That is, there is a specificity of tissue type, from organ to organ in a strain, which will make these organs react in a given way to a given type of irritation.

12. It is, therefore, possible for ancestry to transmit to its posterity every possible combination of the neoplastic or leukemic tendencies which they carry either actually or potentially.

13. This specificity of tissue type in organs, which will insure its reacting in a given neoplastic manner or accepting leukemic deposits, in response to a given chronic irritation, is what we find to be the nature of cancer heredity, and it is obvious that it will make no difference in the nature of the response of such specific organ tissue, whether the lesion is primary or secondary in that organ or organs.

Heredity, therefore, of a specific type of organ tissue, is here shown to be the fundamental influence in determining the incidence and location of secondary tumors and of leukemia and pseudoleukemia, just as it is in determining, the incidence and location of primary neoplasms.

It is also pointed out that any apparent testimony of the frequent occurrence of secondary tumors in man in tissues where primary tumors rarely occur, would be of no help here, as no human strain has ever been even partially analyzed, and no right conclusions regarding heredity can be drawn except from analyzed strains.

It is also suggested, that until stocks of animals to be used in pathological and bacteriological experiments, have been thoroughly tested out as to their inherited potentialities, such experiments will be lacking in any adequate control, since heredity is not considered although it is tremendously potent.

REFERENCES

(1) Ewing: Neoplastic Diseases, Philadelphia, 1919, p. 86.
(2) Slye, Holmes, and Wells: J. Cancer Res., 1919, iv, 207.
(3) Slye, Holmes, and Wells: J. Cancer Res., 1917, ii, 401.
(4) Slye, Holmes, and Wells: J. Cancer Res., 1921, vi, 57.
(5) Slye, Holmes, and Wells: J. Cancer Res., 1917, ii, 14.
(6) Loeb, L: Univ. Penn. Bull., 1906, xix, 113.
(7) Lewin: Ztschr. Krebsforsch., 1908, vi, 273.
(8) Bashford, et. al: Third Sci. Report, Imperial Cancer Research Fund, 1908, 322.
(9) Woglom: J. Cancer Res., 1918, iii, 47.
(10) LeCount: Reference in J. Cancer Res., 1917, ii, 6.
(11) Ewing: Neoplastic Diseases, Philadelphia, 1919, p. 143.

THE ROUTE AND RATE OF ABSORPTION OF SUBCUTANEOUSLY INJECTED SERUM

IN RELATION TO THE OCCURRENCE OF SUDDEN DEATH AFTER INJECTION OF ANTITOXIC HORSE SERUM *

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Most of the recorded cases of sudden death with symptoms of anaphylaxis which have resulted from injection of foreign proteins in man have followed subcutaneous injections. Furthermore, in most cases, the onset of the symptoms, and often the fatal termination, occurred a very few minutes after the injection. However, it should be impossible to produce death by subcutaneous injection of serum in ordinary amounts, because the normal rate of absorption from the subcutaneous tissues is very slow, and it is known from experimental anaphylaxis that in order to produce acute anaphylactic death the antigen must get into the circulation rapidly. This apparent discrepancy between fact and theory has been made the subject, at the suggestion of Dr. H. Gideon Wells,[1] of the investigation reported here.

In the sensitized guinea-pig, which seems to be the most highly sensitive animal to anaphylaxis known, it is difficult to produce acute anaphylactic death by a subcutaneous injection of antigen. Paul Lewis[2] found that from 6 to 10 c.c. of serum injected into a sensitized guinea-pig would not kill, and from the way the animals behaved he calculated that from 12 to 20 c.c. would be required to kill a sensitized guinea-pig, when injected subcutaneously. Rosenau and Anderson[3] injected 6 c.c. of serum subcutaneously into a series of sensitized guinea-pigs, and several of them died in

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1. This work was begun several years ago (1914-15), but on account of the war and other interruptions, its completion has been delayed until this time.

2. Lewis, Paul: J. Exper. Med. **10**: 1, 1908.

3. Rosenau and Anderson: Bull. 45, Hyg. Lab., U. S. P. H. S., 1908.

acute shock. In a series of experiments outlined in the accompanying table I found 15 c.c. to be the smallest amount which, when injected subcutaneously, would cause death of sensitized guinea-pigs. If we take 6 c.c. as the minimum subcutaneous dose that will kill a 250 gm. guinea-pig, in proportion to body weight nearly 2 liters of serum would be required to kill a 70 kg. man, even if man could be as highly sensitized as a guinea-pig; and it is believed that he is not so reactive. Nevertheless, in spite of the difficulty of killing even the most readily sensitized animal known with a subcutaneous injection of antigen, a considerable number of cases of sudden death from anaphylaxis in man following the subcutaneous injections of horse serum have been described.

Rosenau and Anderson,[4] in their pioneer work on anaphylaxis, collected nineteen such instances from the literature, and state that they know personally of many more. Gillette[5] collected twenty-eight cases. Since these papers appeared, reports of sudden acute anaphylactic death occasionally appear in the literature. What Gillette has said about the instances that he collected appears to be true of all. "Practically all these cases have symptoms in common. In a few minutes after receiving the injection they are seized with an intense dyspnea, followed by edema and urticaria. The action of the heart continues long after respiration has ceased. . . . When death terminated the reaction, only two cases lived longer than ten minutes after receiving the serum." Indeed, it seems true that the sooner any serum reaction appears after injection, the more severe it is. Wiley[6] describes a case of a man, aged 34, who was given a prophylactic dose of 1,000 units of antitoxin subcutaneously, 4 inches above Poupart's ligament. He suffered no pain, but spoke of the "lump that raised" when the needle was withdrawn. He remained in his chair from two to three minutes, and then began to scratch his head vigorously, complaining of not being able to breathe. Breathing became more and more labored, and he died in not more than five minutes after the injection.

4. Rosenau and Anderson: Bull. 29, Hyg. Lab., U. S. P. H. S., 1906.
5. Gillette: Therap. Gaz. **32**: 159, 1909.
6. Wiley, S. N.: Sudden Death After Injection of Diphtheria Antitoxin, J. A. M. A. **50**: 137 (Jan. 11) 1908.

Boone [7] related that a boy, aged 10 years, with diphtheria, was given 4,000 units of antitoxin below the shoulder blade. Just as the syringe was laid down, the boy cried out and choked. He suffered difficulty in breathing and convulsions. He finally stopped breathing, and in from five to six minutes after the injection, he was dead. Von Pirquet and Schick,[8] in their monograph on serum disease, speak about a case they saw in which an immediate reaction followed subcutaneous injection of serum. From fifteen to twenty minutes after injection, the patient went into collapse and almost died, but later recovered. Another case is described [9] of a 5-year-old boy who was given a prophylactic dose of diphtheria antitoxin in the scapular region. Before the injection he was completely well. About five minutes after injection, the physician was recalled to find the child dead.

These cases of fatal anaphylaxis are inconsistent with what is known about the rate of absorption from the subcutaneous tissues, and the rate of appearance of antigen in the circulation in the amount necessary to produce death in a highly sensitized animal. Besides being proved by the bulk of clinical experience, the slow absorption of serum from the subcutaneous tissues has also been proved experimentally by testing for the time of appearance of horse serum in the circulation of an animal which has received a subcutaneous injection of this serum.

J. H. Smith [10] injected serums containing agglutinin for *B. coli*, and antitetanolysin and antitoxin for diphtheria, subcutaneously into goats, rabbits and man. By testing samples of blood drawn at different intervals after injection, he found that at the end of five hours in the case of the antitoxin only one tenth of the amount injected had been absorbed, and that the maximum amount in the circulation was not reached until from two to three days after injection. W. H. Park [11] finds that after the subcutaneous injection of diphtheria antitoxin, only 2 units per cubic centimeter of blood are found in the circulation, while after intra-

7. Boone, E. L.: Sudden Death Following Use of Diphtheria Antitoxin, J. A. M. A. **50**: 453 (Feb. 8) 1908.
8. Von Pirquet and Schick: Die Serumkrankheit, Leipzig, Deuticke, 1905.
9. A note in Medical News, Aug. 11, 1906.
10. Smith, J. H.: J. Hyg. **7**: 709, 1907.
11. Park, W. H.: Boston M. & S. J. **168**: 71, 1913.

venous injection the same amount of blood after the same length of time contains 20 units. Lemaire [12] found by means of the precipitin reaction that at the end of two hours after a subcutaneous injection of antitoxin there was only a trace of horse serum in the circulation. It was most abundant at the end of twenty-four hours. Practically the same results were obtained in experiments by Pfeiffer and Friedberger,[13] Bullock,[14] and Morgenroth and Levy.[15]

Since guinea-pigs can be sensitized with extremely small amounts of horse serum, it was thought that the anaphylaxis reaction would be admirably suited for study of the rate of appearance of horse serum in the circulation after subcutaneous injection. It has been the method of choice with investigators to test an unknown substance for the presence of a suspected protein. Accordingly, in order to use the method for this purpose, a series of dogs was injected subcutaneously with various amounts of normal horse serum. At intervals after the injection, samples of the blood and lymph were injected into guinea-pigs. After a period of incubation these animals were reinjected with horse serum to test their sensitization by this substance, which might have been present in the blood and lymph of the dogs. However, even when it was more than probable that enough horse serum had been absorbed into the circulation of the dogs to produce sensitization of the guinea-pigs, negative results were always obtained. It was found that when definite amounts of horse serum were deliberately added to samples of dog serum, and injected into guinea-pigs, sensitization to the horse serum was not produced. As much as 0.1 c.c. in 10 c.c. of dog serum would not sensitize, even though 0.000001 c.c.[4] of horse serum alone will produce a high sensitization. Not only will dog serum inhibit sensitization to horse serum, but other serums (human, cat, beef) will do likewise, and these serums will also inhibit sensitization to dog serum if the proportions are reversed. Further experiments showed that not only serums but also solutions of pure proteins (egg albumin) in the proper amounts will

12. Lemaire: Compt. rend. Soc. de biol. **60**: 578, 1906.
13. Pfeiffer and Friedberger: Zentralbl. f. Bakteriol. u. Parasitenk, I, **37**: 131, 1904.
14. Bullock: J. Path. & Bacteriol. **5**: 274, 1898.
15. Morgenroth and Levy: Therapeut. Monatsh. **23**: 6, 1909.

inhibit sensitization to another protein. The analysis of these phenomena and a theory for their explanation form the subject matter of a paper previously published.[16] In it is suggested that in the animal body the number of receptors that will take up a protein as antigen are limited, and when two antigens are injected in very unequal proportions the antigen in the largest amount will saturate most of the receptors to the extinction of available receptors for the smaller amount. Since the amount of antibody production is proportional to the number of receptors affected, the effect of the antigen injected in the smaller amount is almost completely obscured.

While the anaphylaxis reaction was proved to be worthless for demonstrating the presence of small amounts of horse serum or other protein in the serum of another animal, the complement fixation reaction was found to be ideally suited for the purpose. The presence of foreign proteins does not interfere with it. As small an amount as 0.0001 of a cubic millimeter in 0.5 c.c. of dog serum can be plainly detected.

Having shown that an extremely small amount of horse serum can be detected when added to dog serum, it was sought to determine whether it can be detected in the same proportion in the circulating blood.

A dog weighing 15.8 kg. was used. According to the method of Welcker,[17] a dog of this weight has 1,216 c.c. of blood (7.7 per cent. of body weight). A 1 : 5,000 solution of horse serum was made, and 7.5 c.c. of this was injected intravenously into the dog. This amount of horse serum gave approximately a concentration of 1 : 1,000,000 in the dog's blood. After an interval of four minutes to allow for thorough mixing, a sample of blood was withdrawn from a leg vein of the dog. The serum was tested with the complement fixation test for horse serum. Although there was not complete inhibition of hemolysis, a sufficient inhibition was found to be called a positive reaction.

A dog weighing 12 kg. was injected with 5 c.c. of a 1 : 500 solution of horse serum directly into the blood stream. This amount of horse serum gave approximately a 1 : 100,000 dilution of horse serum in the dog's blood. The serum from a sample of blood withdrawn four minutes after injection gave an almost complete inhibition of hemolysis when tested with the complement fixation test for horse serum.

The relation of the concentration of foreign serum that must be present in the blood of an animal, in order

16. Lewis, J. H.: J. Infect. Dis. **17**: 241, 1915.
17. Welcker: Ztschr. f. rat. Med. **1**: 137, 1858.

that it can be detected by the complement fixation reaction, to the concentration that must be present to produce anaphylactic shock, is of importance in this connection. According to my experiments, complement fixation detects a concentration of horse serum in dog serum of from 1 : 100,000 up to 1 : 1,000,000. The minimum fatal intravenous dose of horse serum for a sensitized guinea-pig (from 0.00001 to 0.000001 c.c.) represents a concentration of from 1 : 200,000 up to 1 : 2,000,000, or approximately the same concentration as the amount detectable by complement fixation.

In order to determine the normal rate of absorption of horse serum from the subcutaneous tissues, a dog, after the removal of a sample of normal blood, was injected subcutaneously with 10 c.c. of horse serum and, at various intervals up to twenty-four hours after injection, samples of blood were taken. These samples, with the normal sample, were tested by the complement fixation test for horse serum. The results show that detectable amounts of horse serum first appear in the circulation one and one-half hours after injection and are present for the entire twenty-four hours. This result confirms those obtained by investigators with other methods, in that it shows that horse serum is absorbed from the subcutaneous tissues slowly and that it does not appear in the circulation rapidly enough to account for the acute anaphylactic deaths and the severe reactions which occur suddenly in men after antitoxin administration.

The reason that subcutaneous absorption is slow is probably that it takes place through the lymphatics, where the movement of the lymph is much slower than that of the blood. A review of the literature on the route of absorption from the subcutaneous tissues shows conflicting opinions as to whether it is lymphatic or vascular. All the experiments described were made with easily diffusible substances. Because of the confusion that exists, and since none of the experimenters used serum, a study of the route of subcutaneous absorption was made by means of the complement fixation test.

A dog under ether-morphin anesthesia was injected subcutaneously with horse serum. Samples of lymph taken from a cannula in the thoracic duct (the duct being excluded from connection with the vein), and of blood from a leg vein taken at various intervals after injection, were tested with the com-

plement fixation test, horse serum being used as antigen. It was found that the first trace of horse serum appeared in the lymph forty minutes after the injection, and in the blood three and one-half hours after injection.

The experiment indicates that the main route of absorption is by the lymphatics, but that probably some absorption takes place through the blood vessels. However, it may be that the presence of horse serum in the blood can be explained by some connection between the lymphatics and the blood vessels other than at the mouth of the thoracic duct.

There must be, then, factors other than extreme sensitization to explain the occurrence of sudden death and immediate severe reactions after subcutaneous injections in man, because, even if extreme sensitization existed, the absorption from the subcutaneous tissues is not rapid enough to meet the requirements for a fatal or a severe reaction. Whatever these factors are, they must operate to produce the rapid appearance of the horse serum in the circulation. It is a common practice among physicians to massage the wheal produced by serum injections in order to promote absorption. It is possible that this procedure forces the serum from the subcutaneous spaces into the lymphatics, and therefore it reaches the circulation much sooner than by the usual process of absorption. In order to test this possibility, a series of guinea-pigs was sensitized by an intraperitoneal injection of horse serum; after a period of incubation they received various doses of horse serum subcutaneously, and the effect of massaging the site of injection was observed. The results are seen in the accompanying table.

It is shown that a dose of horse serum which is not large enough to kill a sensitized guinea-pig when injected subcutaneously will kill quickly when the site of injection is vigorously massaged. The only feasible explanation of this fact is that the massage forces the serum into the circulation through the lymphatics. Another experiment designed to test the same idea is as follows:

A dog under anesthesia was prepared so as to obtain samples of lymph from the thoracic duct and of blood from a leg vein. Horse serum (10 c.c.) was injected subcutaneously on the abdomen, and the site of injection was immediately massaged vigorously. A sample of blood and lymph was taken imme-

diately and at intervals of five minutes thereafter. There was a positive test for horse serum in the sample of lymph collected during the interval fifteen or twenty minutes after injection, and in the blood two hours after injection.

When compared with a similar experiment without massage described above, this experiment indicates that massage hurries the appearance of the horse serum in the circulation.

A further proof that substances can be forced into the lymphatics is the method which anatomists use in injecting the lymphatics of the subcutaneous tissues.

EFFECT ON THE PRODUCTION OF ANAPHYLACTIC SYMPTOMS IN SENSITIZED GUINEA-PIGS OF MASSAGE OF THE SITE OF INJECTION OF THE SECOND DOSE OF ANTIGEN

Guinea-Pig.	First Injection C.c.	Second Injection of Horse Serum, 17 Days Later, Subcutaneously C.c.	Results
1	0.1	1	Showed slight symptoms; recovered
2	0.1	1	Showed slight symptoms; recovered
3	0.1	5	Showed definite symptoms; recovered
4	0.1	5	Showed very severe symptoms; recovered
5	0.1	10	Showed definite symptoms; recovered next morning
6	0.1	10	Showed very acute symptoms; recovered in one hour
7	0.1	15	Showed symptoms immediately; dead next morning
8	0.1	15*	Dead in five minutes
9	0.1	20	Showed acute symptoms immediately. Respiratory convulsions; recovered next morning
10	0.1	20*	Dead in forty-five minutes

* Massaged.

A heavy substance like mercury is forced under the skin under high pressure. When sections are made of such an area, the lymphatics are found clearly outlined, owing to the presence of the easily stained mercury which was forced through the tissues along the lymphatics. An experiment designed to imitate this experiment of the anatomists was carried out as follows:

A large dog was anesthetized and prepared as in the preceding experiment so that samples of thoracic lymph and blood could be obtained at various intervals. About 200 c.c. of horse serum was forced from a pressure bottle, by means

of compressed air, into the subcutaneous tissues of the abdomen. Such an injection produced a large board-hard lump about the size of an ordinary orange. The samples of blood and lymph were tested for horse serum by the complement fixation test. The sample of lymph obtained the first five minutes after injection gave a complete inhibition of hemolysis. The serum of the blood taken forty minutes after the injection showed the first traces of horse serum.

The effect of the extremely high pressure is clearly shown in the immediate appearance of horse serum in the lymph and its early appearance in the blood.

Although certain procedures can hasten the appearance of horse serum injected subcutaneously into the circulation, it is certain that in most cases they are not practiced. The usual history in fatal cases is that symptoms appear just after injection is made, usually before there is an opportunity for manipulation. It is very probable that in these unfortunate cases the injections are accidentally made into the blood stream. The subcutaneous tissues contain numerous blood vessels of good size, and it is quite possible for the point of the hypodermic needle to enter one of these vessels, emptying the contents of the syringe into the circulation. In order to produce death only a small amount need be injected into the circulation of a highly sensitized individual. Boughton [18] reports a fatal reaction following immediately after intravenous injection of 1 minim of horse serum into a man with horse asthma. The possibility of an accidental intravenous injection is made more probable by the experiences obtained after the subcutaneous injection of oils and fats. Fat embolism has occurred after such injections, and any possible cause of the fat embolism, other than accidental intravenous injection, apparently can be ruled out. Moeller [19] reports fat embolism occurring forty-three times in 315 patients injected subcutaneously with oily substances 3,835 times. Ebstein [20] found fat embolism seven times in 908 patients injected 8,292 times. Febriger [21] reports a case in which death followed subcutaneous injections of olive oil for nourishment made necessary because of a stricture of the esophagus. On the twentieth day of injection the

18. Boughton, T. H.: Anaphylactic Death in Asthmatic, J. A. M. A. **73**: 1913 (Dec. 27) 1919.
19. Moeller: Arch. f. Dermat. u. Syph. **37**: 431, 1896.
20. Ebstein: Allg. med. Centralbl., 1896, p. 30.
21. Febriger, quoted by Hodges: Med. Record (Am.) **50**: 76, 1903.

patient coughed violently while the injection was being made, became cyanotic, suddenly collapsed, and became unconscious. In three hours he developed hemiplegia. The postmortem examination revealed fat emboli distributed all over the body. Heidingsfeld [22] emphasizes the fact that fat embolism occurs very often from paraffin prosthesis and from injections of mercury in oily menstruums.

The probability of accidental intravenous injections as the cause of sudden death emphasizes the need of care to ascertain whether a blood vessel is injured by the needle before a subcutaneous injection is made. Before the injection begins, an aspiration of the subcutaneous tissues should be made in order to see whether blood can be pulled into the syringe.

CONCLUSIONS

The complement fixation test can be used to detect extremely small amounts of one serum in the presence of larger amounts of another.

The absorption of serum from the subcutaneous tissues is very slow.

The main route of absorption from the subcutaneous tissues is through the lymphatics.

The rate of absorption of horse serum injected subcutaneously is apparently too slow to explain the cases of sudden anaphylactic death which have occasionally followed the administration of antitoxic serums in man.

Certain procedures, such as massage of the site of injection, and the injecting of large volumes under high pressure, can hasten the appearance in the circulation of horse serum injected subcutaneously.

Because of the suddenness with which it appears, and because of the analogy to the occurrence of fat emboli after the subcutaneous injection of oily substances, acute anaphylactic death in man following subcutaneous injections of horse serum is probably due to an accidental intravenous injection of the serum.

The need of care to exclude as far as possible the injury of a subcutaneous blood vessel during subcutaneous injection of horse serum is indicated.

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22. Heidingsfeld: J. Cutan. Dis. **24**: 513, 1906.

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The Composition of Appendiceal Concretions

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THE COMPOSITION OF APPENDICEAL CONCRETIONS*

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Concretions of varying appearance, density and structure are often found in the human appendix, both with and without inflammatory changes in the organ itself. Owen T. Williams [1] has made a particular study of these, calling attention to the fact that the appendix seldom contains fecal material, and that the usual appendiceal stones are not fecal concretions but concentrically laminated concretions, often larger than the lumen of the appendix between the stone and the cecum, and therefore presumably formed by secretion from the wall of the appendix. According to him, the typical appendix concretion is almost white, with a smooth cut surface resembling soap, whereas fecal concretions are pigmented and have a greasy appearance. Like the intestinal sand, they contain fats soluble in ether and soaps insoluble in ether. He analyzed three appendix concretions, and found material soluble in ether to the amount of 14.97, 16.2 and 7.1 per cent., respectively. The fatty acids of soaps insoluble in ether amounted to 15.6, 2.1 and 1.2 per cent., and the amount of calcium was 3.3, 3.6, and 3.2 per cent. He asserts that the calcium exists as soaps, but presents no chemical evidence to establish this opinion.

Bertha Anthony [2] reported the microscopic study of a large number of normal and diseased appendixes, and found material staining pink with scarlet R, sometimes associated with material staining black with silver nitrate, which she also interprets as proving the presence of calcium soaps. As one of us [3] has pointed out, however, silver nitrate does not stain calcium, but indicates the presence of phosphates or chlorids. If a calcium-containing material stains with silver nitrate it usually means the presence of calcium phosphate, and not the presence of calcium soaps. Therefore, the work of Williams and Anthony furnishes no evidence that calcium soaps are formed in the wall of the appendix, or that such soaps are excreted into the lumen of the appendix to form concretions. Nevertheless, it is highly probable that calcium

*From the Otho S. A. Sprague Memorial Institute, and the Department of Pathology, University of Chicago.

1. Williams, Owen T.: Biochem. J. **2**:395, 1907; Brit. M. J., July 27, 1907.
2. Anthony, Bertha: J. M. Res. **25**:359, 1911.
3. Wells, H. Gideon: J. M. Res. **14**:491, 1906.

soaps are present in the appendix, since fats as well as fat-splitting enzymes are present in the bowel, and calcium is excreted largely by way of the intestinal mucosa.

ANALYSES IN THE LITERATURE

We have found but two other analyses of appendiceal concretions in the literature.

Roeser [4] examined a concretion weighing 0.56 gm. which had perforated the wall of the appendix and was found free in the peritoneal cavity. It contained 77.2 per cent. of inorganic matter and 11.4 per cent. fatty substance, which were not further examined, quantitatively. Cholesterol, bile pigments and bile acids were missing, the fatty substance was chiefly palmitin, and the inorganic matter consisted almost entirely of magnesium ammonium phosphate, calcium phosphate and calcium carbonate.

Harley [5] analyzed a friable concretion, weighing 1.2 gm., found in an appendix at an operation for appendicitis. Preliminary examination showed organic débris, mostly sclerous vegetable structures, a few granules of silicates, occasional trichocephalus ova, and the absence of bile pigment, cholesterol, bile acids or uric acid. Quantitative analysis of the air dried concretion showed 13.16 per cent. water, 64.61 per cent. ash, and 22.23 per cent. organic matter and material volatilized in ashing. The ash was calculated to contain, in per cent. of the dry matter, magnesium ammonium phosphate, 4.78, calcium phosphate, 63.61, calcium carbonate, 7.54 per cent.; iron and silica, traces. In the organic material was 6.3 per cent. fatty acids. No attempt was made to determine whether any of the fatty acids were combined as calcium soaps. Evidently this concretion was similar in composition to ordinary calcification deposits in necrotic tissues, such as have been described in and about the appendix,[6] and probably represents the calcification of a healed appendiceal abscess, since the stone is said to have been found "au milieu d'adhérences," and is not at all similar to the true appendiceal stones, which Williams found to contain but little calcium.

AUTHORS' TESTS

Dr. E. R. LeCount has kindly placed at our disposal for analysis a collection of appendiceal concretions found in appendixes removed at operation, at St. Luke's Hospital, Chicago, and collected during the routine examination of these appendixes in the laboratory. The stones were received preserved in a very little weak alcohol, in three

4. Roeser: J. de pharm. et de chim. **4**:251, 1896.
5. Harley: J. de pharm. et de chim. **2**:433, 1910.
6. Rowan and Wells: Surg., Gynec. & Obst., Oct., 1906, p. 503.

separate lots, of from five to ten stones each; and, to secure information as to the average composition of fecal concretions, the stones in each of the three lots were analyzed together. The masses were generally 5 to 10 mm. in diameter, and for the most part dark brown, only two stones (in Group 2) being made up of concentric layers of a white soapy substance, like the concretions analyzed by Williams. When dried to a constant weight at 100 C., the three collections weighed (1) 4.1413 gm., (2) 3.0049 gm., (3) 5.5842 gm.

The material of each lot after pulverizing was extracted by shaking with 150 c.c. of cold water, and the fatty acids of the water-soluble soaps liberated from these water extracts by acid, were determined.

TABLE 1.—Fatty Acids of Water-Soluble Soaps Present in Collection of Appendiceal Concretions

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Fatty acids (gm)	0.0090	0.0079	0.0098
Dry weight (per cent.)	0.21	0.20	0.17

A small amount of calcium and magnesium were dissolved in water. The percentages of dry weight of these water-soluble constituents are presented in Table 2.

TABLE 2.—Water-Soluble Calcium and Magnesium Present in Appendiceal Concretions

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Calcium	0.021	0.07	0.026
Magnesium	traces	.....	.....

The residue from the water extraction, after drying, was extracted with absolute ether in a Soxhlet extractor. The extracts thus obtained amounted in per cent. of total dry weight to 20.02 per cent. in Group 1; 17.87 per cent. in Group 2; 15.83 per cent. in Group 3.

The ether extracts were then extracted with hot acetone. The acetone soluble portions contained cholesterol, koprosterol, coloring matter and presumably other lipins in small amounts. The cholesterol was recrystallized from 90 per cent. hot alcohol. It gave the Salkowski and Pettenkofer reactions, and was determined quantitatively by the digitonin method. The koprosterol was crystallized from 85-90 per cent. alcohol and showed the slow development of the orange-red color on addition of chloroform and sulphuric acid which differentiates koprosterol from cholesterol (Abderhalden). The values obtained in percentage of total dry weight are presented in Table 3.

The acetone-insoluble portions were treated with cold ether, which removed the color almost completely and probably some lecithin, since it gave Pettenkofer's reaction (sulphuric acid and sugar). The residue from the cold ether was dissolved entirely in hot amyl alcohol, which dissolves calcium soaps [7] as well as sodium, ammonium and potassium soaps. Soaps were salted out of the water emulsions of these extracts and the melting points of their fatty acids determined. Table 4 shows the amounts of soaps present, calculated in percentages of dry weight, and the percentage amounts of calcium found.

Evidently, then, very little of the soap present in this fraction could have been calcium soaps. The soaps were saponified and the free acids extracted with ether. These fatty acids had the following melting points: Group 1, 49 C.; Group 2, 52 C.; Group 3, 57 C., indicating that they are chiefly mixtures of stearic and palmitic acids with some impurities.

TABLE 3.—Cholesterol, Koprosterol and Lipins in Appendiceal Concretions

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Cholesterol	1.6	1.7	0.87
Koprosterol	9.6	14.6	9.1
Lipins (by difference)	0.19	0.22	0.13

TABLE 4.—Soaps Present in Appendiceal Concretions

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Amyl alcohol soluble soaps	7.8	0.34	5.3
Calcium	0.016	0.03	0.001

The residue from the first ether extraction was extraced with boiling amyl alcohol and the extracts analyzed for calcium and magnesium. The figures presented in Table 5 give the percentages of calcium and magnesium of the total dry weight soluble in amyl alcohol.

The calcium soaps, calculated as calcium stearate from these calcium values, would amount to these percentages of the original dry material: Group 1, 1.1 per cent.; Group 2, 8.9 per cent.; Group 3, 2.56 per cent.

The percentages of original materials extracted with hot amyl alcohol were: Group 1, 38 per cent.; Group 2, 21.9 per cent.; Group 3, 38.8 per cent.

The residue from the first ether extraction was extracted with boiling 1 per cent. nitric acid, and the determinations presented in Table 6 made. Results are given in percentage of original dry concretion.

7. Wells, H. Gideon: J. M. Res. 1906, **14**:359, 1906.

TABLE 5.—Calcium and Magnesium Soluble in Amyl Alcohol

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Calcium	0.22	1.26	0.36
Magnesium	0.005	0.006	0.007

TABLE 6.—Inorganic Constituents

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Carbon dioxid	0.84	0.85	0.51
Calcium	6.3	13.7	5.01
Magnesium	0.83	1.6	1.19
Phosphorus	3.6	7.51	3.17
Ammonium	0.4	0.35	0.38
Ferric iron	0.08	0.2	0.11

These elements may have been combined as shown in Table 7, in per cent. of original materials.

TABLE 7

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Calcium phosphate	13.02	27.17	7.72
Magnesium ammonium phosphate	4.73	9.18	6.82
Calcium carbonate	1.91	2.05	1.18
Calcium—excess as sulphate or other salts	4.6	2.4	2.7

TABLE 8.—Results of Chemical Analysis of Appendix Concretions*

Groups	1	2	3
	Per cent.	Per cent.	Per cent.
Inorganic material	24.	41.	18.
Calcium phosphate	13.02	27.17	7.72
Calcium carbonate	1.91	2.05	1.18
Magnesium ammonium phosphate	4.73	9.18	6.82
Calcium (undetermined)	4.6	2.4	2.7
Ferric iron	0.08	0.2	0.11
Fatty acids liberated from water-soluble soaps	0.21	0.20	0.17
Total substances soluble in fat solvents	58.23	39.07	54.80
Substances soluble in ether	20.02	17.87	15.83
Cholesterol	1.6	1.7	0.87
Koprosterol	9.6	14.6	9.1
Soaps soluble in both ether and amyl alcohol	7.8	0.34	5.3
Other lipins	0.19	0.22	0.13
Substances soluble in amyl alcohol and insoluble in ether, probably soaps	38.	21.9	38.8
Calcium soaps calculated as calcium stearate	1.1	8.9	2.56
Organic residue (not analyzed)	17.	20.	26.

*Figures represent percentages of the original dried material.

The percentage of total calcium found in all forms was: Group 1, 6.55 per cent.; Group 2, 15.06 per cent.; Group 3, 5.37 per cent.

SUMMARY

The composition of three collections of concretions found in human appendixes was found to vary somewhat, but in general there was a similarity in the makeup of each lot of concretions (Table 8). They contain about half their weight soluble in fat solvents, this material being chiefly soaps, but with considerable koprosterol and a little cholesterol. Of the soaps but a very small proportion seems to have been calcium soaps. About one fourth of the total weight was composed of inorganic material, chiefly calcium phosphate. About one fifth of the material was organic residue, mostly vegetable fiber, indicating that some part of the appendiceal concretions, at least, comes from the cecum. Probably the rest of the concretions is deposited from the walls of the appendix, since the bowel secretions are known to contain much fatty material and calcium.

We wish to express our indebtedness to Dr. E. R. LeCount for the opportunity to make this study, through his generous gift of the necessary materials.

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STUDIES ON PROTEINOGENOUS AMINES.

XII. THE PRODUCTION OF HISTAMINE AND OTHER IMIDAZOLES FROM HISTIDINE BY THE ACTION OF MICROORGANISMS.

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INTRODUCTION.

In a series of papers published in 1919[1] we communicated a method for the microchemical colorimetric estimation of imidazole derivatives and for the quantitative separation of histamine from histidine. These methods enabled us to study the metabolism of histidine under various conditions. Experiments

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497, 521, 539.

with a colon bacillus, isolated from a case of cystitis, gave results that led us to the following conclusions:

1. When the colon bacillus is allowed to metabolize histidine, either alone or in the presence of nitrates or ammonium salts, histamine is not formed.

2. In a medium containing histidine and glycerol, but no nitrates or ammonium salts, histamine is not formed. In this case imidazole propionic acid appears to be formed; but only when the bacillus is forced to grow anaerobically.

3. In a medium containing histidine, glycerol, or glucose and a source of nitrogen, either KNO_3, NH_4Cl, or both, about 50 per cent of the histidine is converted into histamine in the course of 2 weeks when oxygen is present. In the absence of atmospheric oxygen, this and all the other metabolic activities of the bacillus are greatly reduced, probably because the colon bacillus is an aerobic organism by preference.

4. The production of histamine is always coincident with the production of a medium that is distinctly acid. We believe that the histamine is formed by the bacillus to neutralize the excess of acidity that is simultaneously produced from the glycerol.

5. Contrary to the statement sometimes given in text-books and in the literature that carbohydrates prevent the formation of histamine from histidine, we have found that histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose.

Having established the above facts for one particular colon bacillus, we were then led to a consideration of the following queries:

1. Are all strains of colon bacilli capable of decarboxylating histidine in our standard medium?

2. Are other organisms capable of converting histidine into histamine under identical conditions?

3. How does the addition of amino-acids or peptones to our standard medium containing histidine, influence the production of histamine?

Procedure.[2]

In every case the same number of microorganisms—nine billion[3]—was introduced into 200 cc. of an autoclaved medium

[2] For a detailed description of the method see Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539–555.

[3] The method employed in counting the organisms was that of Breed and Brew (Breed, R. S., and Brew, J. D., *New York Agric. Exp. Station, Techn. Bull. 49*, 1916).

having the following composition: 0.2000 gm. histidine dichloride, 0.2000 gm. ammonium chloride, 0.1000 gm. potassium nitrate, 0.4000 gm. potassium dihydrogen phosphate, 0.800 gm. sodium chloride, 0.0200 gm. sodium sulfate (anhydrous), 0.4000 gm. sodium bicarbonate, 0.0100 gm. calcium chloride (anhydrous), and 4.00 cc. glycerol dissolved in sufficient distilled water to give a final volume of 200 cc.

The inoculated flasks were then incubated at 37° for 14 days, unless otherwise specified, after which the mixture was forced through a Berkefeld filter. The hydrogen ion concentration of the filtrate was determined colorimetrically (see below for details). The filter was then carefully washed with at least 200 cc. of water. Concentrated sulfuric acid (1.0 cc.) was added to the combined filtrate which was then freed from water by evaporation in a glass dish on the water bath. The syrupy residue was transferred, with distilled water, to a 25 cc. precision cylinder and diluted to exactly 25 cc. Of this test liquid 10 cc. were transferred to a 35 cc. glass-stoppered bottle, treated with 3 gm. of solid sodium hydroxide, and extracted six times with amyl alcohol using 20 cc. for each extraction. This divides the material into two fractions, the amyl alcohol extract, which may contain histamine and methyl imidazole and which we refer to as the histamine fraction, and the alkaline aqueous liquid which contains histidine and may contain imidazole acetic, propionic, lactic, and acrylic acids.

The combined amyl alcohol extracts were extracted with normal sulfuric acid, which removes the imidazoles. The acid extracts were nearly neutralized with 5 N NaOH and the resulting liquid was diluted to exactly 100 cc. The amount of histamine present was then determined colorimetrically by means of the well known reaction that occurs between *p*-phenyldiazonium sulfonate and imidazole derivatives in a solution rendered alkaline with sodium carbonate.[1] When the presence of histamine was indicated by the colorimetric determination, an amino nitrogen determination was also carried out on this fraction. The values obtained by these methods check closely when histidine is the only amino-acid present in the original medium. In other cases the amino nitrogen values are invariably high. To be certain that histamine was the only imidazole present, a methyl imidazole

determination was also carried out. We have never encountered methyl imidazole as a product of the bacterial decomposition of histidine. The presence of histamine was, moreover, qualitatively verified by means of physiological methods.

The histidine fraction was transferred to a 25 cc. graduated precision cylinder with water and 7 cc. of 37 per cent HCl. The cooled acid liquid was then diluted to 25 cc. Portions of this liquid were than tested for histidine, colorimetrically and by means of a Van Slyke amino nitrogen determination. When the values obtained check closely, histidine is probably the only imidazole present. If the color obtained is too red, and the colorimetric determination indicates the presence of considerably more imidazole than can be accounted for as histidine by the amino nitrogen method, the excess color is probably due to imidazole acetic, propionic, lactic, or acrylic acids. Which of these acids is present cannot be determined without an isolation experiment. We have made no effort to isolate these acids but have calculated the excess imidazole value as imidazole propionic acid. When the amino nitrogen determination indicates the presence of considerably more histidine than can be accounted for colorimetrically, we are confronted with two possibilities:

1. Some of the introduced ammonia may have been converted into a carboxylated, alkali-stable, primary amino compound—possibly an amino-acid—which would give off nitrogen with nitrous acid; or

2. Some of the histidine may have suffered a rupture of the imidazole ring with the liberation of free amino groups.

Up to the present time we have brought no absolute proof that either of these is the correct explanation for the facts. At present we are inclined to believe that the second of these possibilities is the most probable and our reason for this belief is outlined in the following pages.

Determination of the Hydrogen Ion Concentration.

In our earlier work we employed the set of standard phenolsulfonephthalein tubes furnished by Hynson, Westcott and Dunning to determine the pH of our media after incubation. This set is inadequate for work of this kind because it is useful only

within the narrow limits of pH 6.6 to 8.6. Most of our final media were too strongly acid to fall within the range of this indicator. We have, therefore, prepared a series of standard tubes with a lower pH value of 1.2 by following the method of Clark and Lubs.[4] The indicators selected were phenol red, range 8.6 to 6.6; brom-cresol purple, range 6.8 to 5.2; methyl red, range 6.0 to 4.4; brom-phenol blue, range 4.6 to 3.0; and thymol blue, range 2.8 to 1.2.

We have found that the buffer solutions containing the sulfonephthalein derivatives can be kept for at least 2 years, and hence probably indefinitely, in a sealed Pyrex glass tube after preservation with thymol. It is possible, therefore, to prepare a permanent set of tubes, similar to those distributed by various concerns, for the phenol red range, for all of the sulfonephthalein derivatives. Methyl red, which is not a sulfonephthalein derivative, deteriorates so rapidly that a set of permanent tubes containing this indicator cannot be prepared in the usual way. We have found, however, that the colors produced with methyl red can be imitated by means of an aqueous solution containing mixtures of Congo red and methyl orange. These thymol-preserved aqueous solutions containing Congo red and methyl orange can then be sealed up and kept in Pyrex glass tubes similar to those used for the other standard sets. They seem to keep indefinitely.

The determinations were carried out as follows. A drop of the filtrate to be tested was transferred to a porcelain test plate and mixed with 1 drop of indicator. This procedure was repeated until a color was obtained that was within the range of one of the indicators. Then 1.0 cc. of the filtrate to be tested was mixed with 0.10 cc. of the proper indicator in a Pyrex test-tube and the color compared with that of the standard tubes. The inherent color of the filtrates was never sufficiently intense to have any effect upon the accuracy of the determination.

Method and Table for the Estimation of Small Amounts of Imidazole Lactic Acid.

In our previous papers[1] we reported tables by means of which colorimetric readings could be converted into milligrams of his-

[4] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1.

tidine, histamine, methyl imidazole, and imidazole acetic and propionic acids. It seemed desirable to have a similar table for the estimation of imidazole lactic acid. This substance was, therefore, prepared using the method described by Fränkel.[5] The perfectly white solid obtained by this method, after two recrystallizations from water, had the following properties.

1. Melting point 217°.
2. Chlorine—none.
3. Residue on ignition—none.
4. Ammonia—none.
5. Amino nitrogen (Van Slyke method)—none.
6. The solid—0.1000 gm.—was dissolved in 10 cc. of 0.10 N NaOH and allowed to react for 1 hour at room temperature. The excess of alkali was determined by titration with 0.10 N HCl, using phenolphthalein as indicator. The first change in the indicator was obtained when 4.3 cc. of the 0.10 N acid had been added, 4.5 cc. of the acid being required to give a colorless solution. The indefinite end-point obtained is exactly what one would expect of a substance having a fairly strong acid group and a feebly basic group. The 5.7 cc. of 0.1 N NaOH used for 0.1000 gm. of substance agrees very well with the 5.74 cc. demanded by theory for $C_6H_8N_2O_2 \cdot H_2O$. We therefore considered the substance to be 100 per cent pure.

A stock solution was prepared by dissolving 0.5000 gm. of the solid in 28.7 cc. of 0.10 N HCl and diluting with water to 50 cc. From this the standard test solution was prepared by diluting 1 cc. to 100 cc. in a volumetric flask. The tabular values were then obtained by mixing different amounts of this standard solution with the alkaline *p*-phenyldiazonium sulfonate reagent as previously described.[1] The color produced was then compared in a Duboscq colorimeter with a standard solution of Congo red.[6]

The color produced matches that of the Congo red solution perfectly. A color of maximum intensity is obtained within 3 to 5 minutes and it is stable for from 5 to 10 minutes during which time an accurate comparison can easily be made. With

[5] Fränkel, S., *Monatsh. Chem.*, 1903, xxiv, 229.

[6] To prepare the Congo red solution, vacuum-dried Grubler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volume flask. Water is then added to the mark. This is the stock solution which keeps indefinitely. From it the standard indicator solution is prepared by diluting 1.00 cc. with distilled water to 500 cc. in a volume flask.

TABLE I.

Estimation of Small Amounts of Imidazole Lactic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole lactic acid ($C_6H_8N_2O_3 \cdot H_2O$) in the test cylinder. (Total volume 8 cc.) Test cylinder set at 20 mm.
mm.	*gm.*
1.0	0.000001
2.0	0.000002
3.0	0.000003
4.1	0.000004
5.1	0.000005
6.1	0.000006
7.1	0.000007
8.1	0.000008
9.2	0.000009
10.2	0.000010
11.2	0.000011
12.2	0.000012
13.2	0.000013
14.3	0.000014
15.3	0.000015
16.3	0.000016
17.3	0.000017
18.3	0.000018
19.4	0.000019
20.4	0.000020
21.4	0.000021
22.4	0.000022
23.4	0.000023
24.5	0.000024
25.5	0.000025
26.5	0.000026
27.5	0.000027
28.5	0.000028
29.6	0.000029
30.6	0.000030
31.6	0.000031
32.6	0.000032
33.6	0.000033
34.7	0.000034
35.7	0.000035
36.7	0.000036
37.7	0.000037

TABLE I—*Concluded.*

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole lactic acid ($C_6H_8N_2O_2.H_2O$) in the test cylinder. (Total volume 8 cc.) Test cylinder set at 20 mm.
mm.	*gm.*
38.7	0.000038
39.8	0.000039
40.8	0.000040
41.8	0.000041
42.8	0.000042
43.8	0.000043
44.9	0.000044
45.9	0.000045
46.9	0.000046
47.9	0.000047
48.9	0.000048
50.0	0.000049
51.0	0.000050

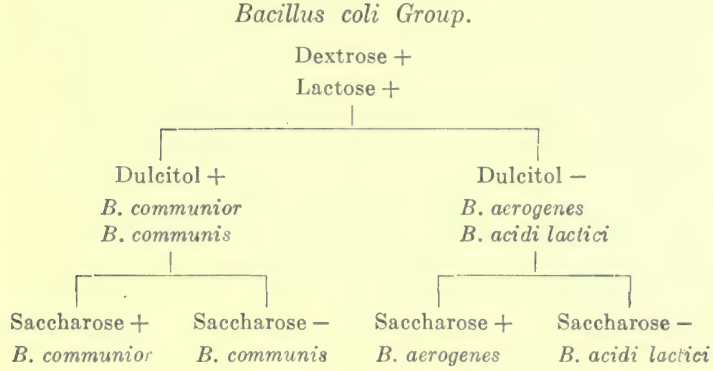
quantities of this acid in excess of 0.000025 gm., the color produced is too intense to enable one to make an accurate comparison. In such cases the test cylinder was set at 10 instead of at 20. The reading obtained was then multiplied by two before subtracting the correction blank, which is 0.30 mm. as in the case of the other imidazoles.[7]

PART I.

On The Products Formed From Histidine by the Action of Bacillus coli.

These four organisms (*Bacillus coli communis, Bacillus coli communior, Bacillus lactis aerogenes,* and *Bacillus acidi lactici*) comprise the colon group (in the narrower sense) according to the classification of the American Public Health Association. This differentiation is based upon the different behavior of these four organisms toward dextrose, lactose, dulcitol, and saccharose and is represented as follows:

[7] This correction blank represents the amount of color that is autogenously produced by the reagent even when imidazoles are absent. See foot-note 1.



The behavior of the organisms investigated by us is summarized in Table II. We have included a large number of tests that were not required for differentiation purposes because we hoped to find a correlation between these reactions and the production of histamine. Such a relationship seems, however, not to exist. The organisms Coli K, H, B, 88, 51, 80, 84, 90, 52, 74, and Schwartz were isolated from human feces and investigated as soon as they had been obtained in pure culture. The other strains were stock cultures obtained from a variety of sources. Some of these strains have been growing on artificial media for years.

Bacillus coli communior.

Of the organisms investigated, eight belonged to this group. Their behavior on our synthetic medium is summarized in Table III.

Coli P–3–19.—This organism did not rupture the imidazole ring to an appreciable extent. Of the histidine originally introduced, 29.7 per cent was converted into histamine and 59 per cent was recovered unchanged. Imidazoles other than histamine were not formed. Of the ammonia originally introduced, 42 per cent was removed by the micro-organisms.

Coli K (red).—There is a sufficient discrepancy between the amino nitrogen and color values for both the histidine and the histamine fractions to suggest a slight rupture of the imidazole ring with the production of primary amino groups. Of the histidine originally introduced, 37.6 per cent was converted into histamine and 50 per cent was recovered unchanged. Imidazoles other than histamine were not formed. Of the ammonia originally introduced, 9.7 per cent was removed by the bacilli.

TABLE II.

Name of strain.	Mobility.	Gram stain.	Colony on endo.	Gelatin slab.	Milk (coagulation of).	Dextrose.	Lactose.	Saccharose.	Galactose.	Levulose.	Maltose.	Raffinose.	Mannitol.	Mannose.	Arabinose.	Xylose.	Dulcitol.	Inulin.	Salicin.	Indole.	Voges-Proskauer.	Histamine production.	Classification.
Coli P-3-19	−	−	Red.	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	*Bacillus communior.*
" K (red)	−	−	"	−	+	+	+	+				+	+				+					+	
" Y	−	−	"	−	+	+	+	+									+					+	
" Jd	−	−	"	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+		−	
" Lac. Aer	−	−	"	−	+	+	+	+									+					−	
" 88	−	−	"	−	+	+	+	+				+	+				+					−	
" 51	−	−	"	−	+	+	+	+				−	+				+					−	
" H (white)	−	−	White.	−	+	+	+	+									+					−	
" cystitis	−	−	Red.	−	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	−	+	*Bacillus communis.*
" Wk	−	−	"	−	+	+	+	−	+	+	+	−	+	+	−	−	+	−	−	+	−	−	
" Hm	−	−	"	−	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+	−	−	
" Cs	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	+	+	−	−	+	−	−	
" K (white)	−	−	White.	−	+	+	+	−				−	+				+					−	
" bovis No. 3	−	−	Red.	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	*Bacillus aerogenes.*
" " " 4	−	−	"	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+		−	
" 80	−	−	"	−	+	+	+	+				+	+				−					−	
" 84	−	−	"	−	+	+	+	+				+	+				−					−	
" 90	−	−	"	−	+	+	+	+				+	+				−					−	

Coli B	−	−	Red.	−	+	+	+	−				−	+				−						−		*Bacillus acidi lactici.*
" H	−	−	"	−	+	+	+	−									−						−		
" P-1-19	−	−	"	−	+	+	+	−	−	+	+	−	+	+	+	+	−	−	−	+	−	−			
" P-2-19	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	+	−	−	−	+	−	−			
" P-4-19	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	−	+	−	−			
" P-5-19	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	−	+	−	+			
" P-6-19	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	−	−	−	−	+	−	−			
" I (K)	−	−	"	−	+	+	+	−	+	+	+	−	+	+	+	+	−	−	−	+	−	−			
" Schwartz	−	−	"	−	+	+	+	−				+	−				−								
" 52	−	−	"	−	+	+	+	−				−	+				−						−		
" 51 (white)	−	−	White.	−	+	+	+	−				−	+				−						−		
" 74	−	−	Red.	−	+	+	+	−				−	+				−						−		

TABLE III—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli P-3-19.	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Match perfect. 96%	0.10 cc. = 5.9 mm. 0.20 " = 11.8 " Match good. 59%	1.18 cc. N_2 at 22° and 745 mm. 0.1327 gm. histidine dichloride. 66.3%
Coli K (red):	0.10 cc. = 10.8 mm. 0.20 " = 21.6 " Match perfect. 108%	0.10 cc. = 5.0 mm. 0.20 " = 10.0 " Match perfect. 50%	1.14 cc. N_2 at 26° and 750 mm. 0.1264 gm. histidine dichloride. 63.2%
Coli Y, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.63 cc. N_2 at 27.5° and 744 mm. 0.1795 gm. histidine dichloride. 89.7%
30 days.	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Match perfect. 74%	0.10 cc. = 6.6 mm. 0.20 " = 13.2 " Match good. 66%	2.46 cc. N_2 at 24° and 748 mm. 0.275 gm. histidine dichloride. 137.5%
Coli Jd.	0.10 cc. = 10.1 mm. 0.20 " = 20.2 " Match perfect. 101%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.55 cc. N_2 at 22° and 743 mm. 0.1738 gm. histidine dichloride. 86.9%
Coli Lac. Aer., 7 days.	0.10 cc. = 8.7 mm. 0.20 " = 17.4 " Match perfect. 87%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. 75%	1.37 cc. N_2 at 16° and 750 mm. 0.1597 gm. histidine dichloride. 79.8%
14 days.	0.10 cc. = 5.2 mm. 0.20 " = 10.4 " Match perfect. 52%	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match good. 45%	1.81 cc. N_2 at 23° and 748 mm. 0.2032 gm. histidine dichloride. 101.6%

* Colors matched against the (CR-MO) standard.

coli communior.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
0.05 cc. = 7.2 mm. 0.10 " = 14.4 " Color develops like that of histamine.	0.048 gm. of histamine in entire test solution. 29.7% of histamine present.	0.46 cc. N_2 at 22° and 748 mm. 0.042 gm. histamine dichloride. 26%	21.0 Hence the ∞ of 15 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.9
0.05 cc. = 9.1 mm. 0.10 " = 18.2 " Color develops like that of histamine.	0.0607 gm. of histamine in entire test solution. 37.6% of histamine present.	0.9 cc. N_2 at 30° and 748 mm. 0.0785 gm. histamine dichloride. 48.6%	32.5 Hence the ∞ of 3.5 cc. of 0.1 N NH_3 used by the microorganisms.	7.4	5.4
0.50 cc. = 4.8 mm. 1.00 " = 9.6 " Color develops like that of histamine.	0.00128 gm. of histamine in entire test solution. 1.98%		20.0 Hence the ∞ of 16 cc. of 0.1 N NH_3 used by microorganisms.	7.3	6.0
0.20 cc. = 4.0 mm. 0.40 " = 8.0 " Color develops like that of histamine.	0.00668 gm. of histamine dichloride in entire test solution. 4.1%		None. Hence the ∞ of 36 cc. of 0.1 N NH_3 used by microorganisms.	7.3	4.6
None.			28.0 Hence the ∞ of 8 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.6
None.			36.7 Hence the ∞ of 0.7 cc. of 0.1 N NH_3 was produced by microorganisms.	7.3	5.8
None.			38.0 Hence the ∞ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	5.4

TABLE III—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli 88.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.3 mm. 0.20 " = 16.6 " Match perfect. 83%	1.62 cc. N_2 at 27° and 746 mm. 0.1775 gm. histidine dichloride 88.8%
Coli 51.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	1.62 cc. N_2 at 27° and 746 mm. 0.1775 gm. histidine dichloride. 88.8%

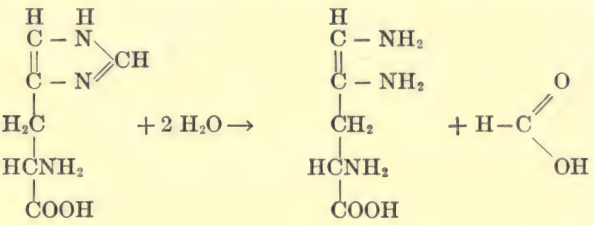
Concluded.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			37.0 Hence the = of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.4
None.			33.75 Hence the = of 2.25 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.4	5.6

Coli Y.—During the first 2 weeks of incubation, 8 per cent of the introduced histidine and 44 per cent of the introduced ammonia were removed by the microorganisms. Of the histidine originally introduced, about 2 per cent was converted into histamine. There is no evidence that the imidazole ring was ruptured because the check between the histidine values by amino nitrogen and colorimetric determinations is perfect.

During the next 16 days of incubation an additional 2 per cent of histamine was produced. The liquid was *free from ammonia;* hence 100 per cent of the ammonia originally introduced was removed by the organisms. The most striking observation, however, was the discrepancy between the histidine values obtained by the colorimetric and the amino nitrogen methods.

In a previous paper we called attention to the fact that histidine might be decomposed by microorganisms according to the following structure:



The triamino compound formed, because of its carboxyl group, would form a sodium salt, in the presence of a strong alkali, that should be soluble in water and difficultly soluble in amyl alcohol. In the course of our standard procedure this triamino compound should appear together with histidine in the histidine fraction. Although histidine contains but one primary amino group, the triamino compound contains three such groups; hence each molecule of triamino compound would give three times as much nitrogen, by the Van Slyke method, as a molecule of histidine.

Going back to the table we find that 90 per cent of the introduced histidine was left unchanged at the end of 14 days. After 30 days of incubation, 66 per cent of histidine was present and 2 additional per cent of histamine had been produced. In all, then, 68 of the 90 per cent of histidine left after 14 days of incubation can be accounted for colorimetrically. What became of the 22 per cent of histidine that cannot be accounted for colorimetrically? If we assume that all of this histidine was converted into triamino compound, an amount of N_2 equivalent to 22 times 3 or 66 per cent of histidine would be evolved in an amino nitrogen determination.

If to this we add the nitrogen evolved by the 66 per cent of histidine present in the same liquid, one would expect to obtain an amount of nitrogen equivalent to 132 per cent of histidine which compares very well with the 137.5 per cent actually obtained.

We realize, of course, that an excess of amino nitrogen may not necessarily indicate the presence of a histidine disruption product. It is possible that non-volatile, carboxylated amino compounds, possibly amino-acids, might be synthesized from ammonia and glycerol, and if these were present, the amino nitrogen figure would be high. At present, however, we are inclined to believe that a triamino compound is responsible for the excess amino nitrogen because a quantitative relationship similar to the one given above has been found to hold in five other cases. A quantitative agreement might be obtained once or twice by accident; but it seems hardly reasonable to assume that the accident should occur six times.

The formula of the triamino compound would lead one to believe that the compound might have some physiological activity. A search of the literature revealed the fact that diamino acetylene derivatives have not yet been prepared. We hope to make the preparation and properties of this triamino compound the subject of a subsequent paper.

Finally we can raise the question, *why do certain microorganisms rupture the imidazole ring with the liberation of free amino groups?* Two reasons suggest themselves. This type of nuclear disruption is the most certain way to expose for future use all of the nitrogen and carbon of the molecule. This is also a decomposition that converts a feebly basic substance into one that is strongly basic.

Coli Jd.—This organism did not rupture the imidazole ring. Of the histidine originally introduced, 86 per cent was recovered unchanged. Histamine and other imidazoles were not formed. Of the ammonia originally introduced 22 per cent was removed by the microorganisms.

Coli Lac. Aer.—During the first 7 days of incubation, this rapidly growing organism reduced the histidine concentration of the solution to 75 per cent of its initial value. There is practically no indication that a nuclear rupture occurred. The concentration of ammonia in the solution was *greater* after 7 days of incubation than it was at the outset of the experiment. Some of this ammonia must have been derived either from the disrupted histidine, or from the potassium nitrate.

The second 7 day period of incubation reduced the histidine concentration to 45 per cent of its initial value. Some of this histidine was apparently converted into triamino compound and some into ammonia. Although there is no doubt, in this case, that a carboxylated amino compound was formed from the histidine, quantitative proof for the formation of a triamino compound is lacking because the decomposition continued beyond this stage with the production of ammonia. Histamine and other imidazoles were not formed.

The increase in the ammonia figure might suggest that this organism was unable to utilize ammonia as a source of nitrogen, and that the imidazole ring was ruptured to render nitrogen available. This was, however, not the case because this organism grows splendidly on a medium that contains only inorganic salts, glycerol, and NH_4Cl. On this histidine-free medium the ammonia value drops from 36 to 25 cc. of 0.1 N HCl in the course of 2 weeks. The rupture of the imidazole ring might, therefore, have been resorted to in an attempt to lower the hydrogen ion concentration of the cell protoplasm.

Coli 88.—This organism did not rupture the imidazole ring with the production of appreciable quantities of a carboxylated amino compound; but some of the histidine nitrogen must have been converted into ammonia because the concentration of ammonia at the end of the 14 day incubation period was greater than the initial concentration. Of the histidine originally introduced, 83 per cent was recovered unchanged. Histamine and other imidazoles were not formed.

Coli 51.—The results obtained with this organism were so nearly like those obtained with Coli 88 that a detailed discussion seems superfluous. In this case, however, the ammonia consumption was more rapid than its production so that the initial ammonia value was reduced by 6.2 per cent.

Bacillus coli communis.

Of the organisms investigated, five belong to this group. Their behavior on our synthetic medium is summarized in Table IV.

Coli cystitis.—This is the organism that was used in our earlier work.[1] Since its behavior has been described in detail in our previous articles, it seems unnecessary to do more here than to call attention to the fact that it has lost none of its power of decarboxylation. In this experiment 57 per cent of the histidine originally introduced was converted into histamine as compared to a 50 per cent conversion obtained by us some 2 years ago.

Coli Wk.—This organism ruptured the imidazole ring to a considerable extent, apparently with the formation of a carboxylated amino compound. Quantitative proof for the formation of a triamino compound is, however, lacking in this case. Of the histidine originally introduced 76 per cent was recovered. Histamine and other imidazoles were not formed. Of the

ammonia originally introduced, 8.3 per cent was removed by the microorganisms.

Coli Hm.—This organism seems not to have attacked the histidine at all because 96 per cent of this amino-acid was recovered. The nitrogen requirements were obviously supplied by the ammonia whose final concentration was only 75 per cent of that originally introduced.

Coli Cs.—This organism did not rupture the imidazole ring with the formation of a carboxylated amino compound. Of the histidine originally introduced, 86 per cent was recovered. Histamine or other imidazoles were not formed. Of the ammonia originally introduced, 25 per cent was removed by the microorganisms.

Coli K (White).—This organism ruptured the imidazole ring to the extent of 8 per cent, apparently with the formation of a triamino compound (see under Coli Y). Of the histidine originally introduced, 92 per cent was recovered. If we assume that the 8 per cent of histidine that disappeared in the course of 2 weeks incubation, was quantitatively converted into triamino compound, the total amino nitrogen value of the liquid should have been eight times 3 plus 92 equals 116 per cent, which compares very well with the 113.4 per cent actually obtained. Histamine and other imidazoles were not formed. A small amount of ammonia was produced either from the histidine or from the potassium nitrate.

Bacillus lactis aerogenes.

Of the organisms investigated, five belong to this group. Their behavior on our synthetic medium is summarized in Table V.

Coli bovis 3.—During the first 2 weeks of incubation, this organism reduced the histidine concentration of the solution to 92 per cent of its initial value and removed 17 per cent of the ammonia that was originally introduced. There is no indication that a nuclear rupture occurred. Histamine and other imidazoles were not produced.

After 30 days of incubation, the histidine concentration was reduced to 84 per cent of its initial value. During this period, 2.4 per cent of histidine was converted into histamine. In all, then, 86.4 per cent of histidine can be accounted for colorimetrically, after 30 days of incubation After 14 days of incubation, 92 per cent of histidine was recovered. If we assume that the 5.6 per cent of histidine that disappeared during the second 16 day period was converted quantitatively into triamino compound, an amino nitrogen value of 5.6 times 3 plus 84 equals 100.8 per cent should have been obtained for the histidine fraction which compares very well with the 103.5 per cent actually obtained. This organism seems, therefore, to have converted 5.6 per cent of histidine into triamino compound. Of the ammonia originally introduced, 28.5 per cent was removed by the microorganisms.

TABLE IV—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli cystitis.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Color developed very rapidly. 112%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. 40%	0.85 cc. N_2 at 20° and 750 mm. 0.097 gm. histidine dichloride. 48.5%
Coli Wk.	0.10 cc. = 8.4 mm. 0.20 " = 16.9 " Match perfect. 84%	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match good. 76%	1.95 cc. N_2 at 22° and 748 mm. 0.220 gm. histidine dichloride. 110%
Coli Hm.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. 103%	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Match perfect. 96%	1.80 cc. N_2 at 20° and 750 mm. 0.206 gm. histidine dichloride. 103%
Coli Cs.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.57 cc. N_2 at 23° and 750 mm. 0.1768 gm. histidine dichloride. 88.4%
Coli K (white).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.2 mm. 0.20 " = 18.3 " Match perfect. 92%	2.07 cc. N_2 at 28° and 750 mm. 0.2268 gm. histidine dichloride. 113.4%

* Colors matched against the (CR–MO) standard.

coli communis.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction. Before incubation.	Reaction. After incubation.
			cc.	*pH*	*pH*
0.05 cc. = 13.8 mm. 0.10 " = 27.5 " Color develops like that of histamine.	0.092 gm. of histamine dichloride in the entire test solution. 57% of histamine present.	1.04 cc. N_2 at 20° and 754 mm. 0.0964 gm. histamine dichloride. 59.8%	27 Hence the = of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.4
None.			33 Hence the = of 3 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	6.4
None.			27 Hence the = of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.2
None.			27 Hence the = of 9 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	5.8
None.			37 Hence the = of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.5

TABLE V—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli bovis 3, 14 days.	0.10 cc. = 10.6 mm. 0.20 " = 21.2 " Match perfect. 106%	0.10 cc. = 9.2 mm. 0.20 " = 18.4 " Match perfect 92%	1.65 cc. N_2 at 27° and 749 mm. 0.182 gm. histidine dichloride. 91%
30 days.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.4 mm. 0.20 " = 16.7 " Match perfect. 84%	1.85 cc. N_2 at 21° and 738 mm. 0.207 gm. histidine dichloride. 103.5%
Coli bovis 4.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. 103%	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	1.93 cc. N_2 at 26° and 745 mm. 0.212 gm. histidine dichloride. 106%
Coli 80.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.97 cc. N_2 at 30° and 747 mm. 0.213 gm. histidine dichloride. 106.5%
Coli 84.	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.79 cc. N_2 at 25° and 751 mm. 0.2000 gm. histidine dichloride. 100%
Coli 90.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.51 cc. N_2 at 21° and 752 mm. 0.172 gm. histidine dichloride. 86%

* Colors matched against the (CR–MO) standard.

lactis aerogenes.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			29.7 Hence the = of 6.3 cc. of 0.1 N NH_3 used by microorganisms.	7.3	5.4
0.40 cc. = 4.7 mm. 0.80 " = 8.4 " Color develops like that of histamine.	0.0039 gm. of histamine dichloride in the entire test solution. 2.4% of histamine present.		25.7 Hence the = of 10.3 cc. of 0.10 N NH_3 used by the microorganisms.	7.3	5.2
None.			36.0 Hence appreciable quantities of NH_3 seem not to have been removed by the microorganisms.	7.4	5.4
None.			29 Hence the = of 7 cc. of 0.1 N NH_3 used by the microorganisms.	7.4	5.4
None.			37 Hence the = of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			37.5 Hence the = of 1.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6

Coli bovis 4.—This organism did not grow as well as the other coli and it seems not to have attacked appreciable quantities of either the histidine or the ammonia.

Coli 80.—This colon bacillus ruptured the imidazole ring with the formation of carboxylated amino compounds. Of the histidine originally introduced, 80 per cent was recovered. Histamine and other imidazoles were not formed. The ammonia concentration was reduced by 19.5 per cent.

Coli 84.—A nuclear rupture with the formation of carboxylated amino compounds is again indicated in this case. Of the histidine originally introduced, 86 per cent was recovered. Some ammonia was produced either from the disrupted histidine or from the potassium nitrate. Histamine or other imidazoles were not formed.

Coli 90.—The liquid finally obtained in this case was free from carboxylated amino compounds other than histidine. Of this amino-acid, 84 per cent was recovered unchanged. Some ammonia was produced either from the disrupted histidine or from the potassium nitrate. Histamine or other imidazoles were not formed.

Bacillus acidi lactici.

Of the organisms investigated, twelve belong to this group. Their behavior on our synthetic medium is summarized in Table VI.

Ten of these organisms gave results that are qualitatively identical; so they can be discussed collectively. Only Coli P-2-19 and P–5–19 require special consideration.

All of the ten similar organisms ruptured the imidazole ring with the production of carboxylated amino compounds. The disruption did not proceed quantitatively to the formation of triamino compound in any of these cases. Histamine and other imidazoles were not formed. Some of the organisms—Coli H, Schwartz, 52, 51 (white), and 74—produced ammonia, and others —Coli P–1–19, P–4–19, P–6–19, and I (K)—removed some from the solution.

Coli P-2-19.—The formation of a carboxylated triamino compound is again quantitatively indicated in this case. Of the histidine originally introduced, 95 per cent was recovered. If we assume that the 5 per cent of histidine that disappeared was quantitatively converted into a triamino compound, an amino nitrogen value of five times 3 plus 95 or 110 per cent should have been obtained which agrees almost exactly with the 110.2 per cent actually obtained. Histamine and other imidazoles were not formed. Of the ammonia originally introduced, 57 per cent was removed by the microorganisms.

Coli P-5-19.—This interesting organism converted 14.5 per cent of the histidine originally introduced, into histamine. Since 79 per cent of histidine was recovered unchanged, 93.5 per cent of the original histidine can be accounted for colorimetrically. The amino nitrogen determination on the histidine fraction gave a value of 100.5 per cent calculated as histidine. Of this amino nitrogen only 79 per cent could have been derived from histidine. If we assume that the remaining 21.5 per cent of amino nitrogen was derived from the triamino compound, this would account for 7.17 per cent of histidine; *i.e.*, 21.5 divided by 3. Summing up, then, we have

79.0	per	cent	as	histidine.
14.5	"	"	"	histamine.
7.17	"	"	"	triamino compound.
100.67	"	"		total recovery.

which is a truly remarkable agreement.

PART II.

The Products Formed from Histidine by the Action of Other Members of the Colon Typhoid Group.

The organisms investigated were *Bacillus enteritidis*, *Bacillus typhosus*, *Bacillus paratyphosus* A (3 strains), *Bacillus dysenteriæ* Flexner, *Bacillus dysenteriæ* Morgan, *Bacillus dysenteriæ* Shiga, *Bacillus fæcalis alcaligenes* I, and *Bacillus fæcalis alcaligenes III*. The behavior of these organisms on our synthetic medium is summarized in Table VII.

B. enteritidis 228.—This organism grew very well on the liquid medium. There is no evidence of a rupture of the imidazole ring after 7 days of incubation although 20 per cent of the introduced histidine disappeared during this time interval. Of the ammonia originally introduced 32 per cent was removed by the microorganisms. Histamine and other imidazoles were not formed.

The attack on the remaining histidine was so intense, during the second 7 day period, that 40 per cent of that amino-acid disappeared. A nuclear rupture occurred with the formation of some carboxylated amino compound and considerable ammonia. Histamine and other imidazoles were not formed.

B. typhosus.—This organism grew poorly on our medium. Histamine and other imidazoles were not formed. Very little acid was produced. Of the ammonia originally introduced, 80 per cent was recovered. The results on the histidine fraction suggest that a small amount of histidine was deaminized because the colorimetric value for histidine ran higher than the amino nitrogen value. The conversion was too slight, however, to be of particular significance.

TABLE VI—*Bacillus*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli B.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.2 mm. 0.20 " = 18.4 " Match perfect. 92%	1.90 cc. N_2 at 28° and 747 mm. 0.208 gm. histidine dichloride. 104%
Coli H.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.80 cc. N_2 at 30° and 747 mm. 0.1945 gm. histidine dichloride. 97.2%
Coli P–1–19.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.1 mm. 0.20 " = 18.2 " Match perfect. 91%	1.70 cc. N_2 at 21° and 744 mm. 0.192 gm. histidine dichloride. 96%
Coli P–2–19.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	1.99 cc. N_2 at 25° and 745 mm. 0.2204 gm. histidine dichloride. 110.2%
Coli P–4–19.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	0.10 cc. = 8.4 mm. 0.20 " = 16.7 " Match perfect. 84%	1.85 cc. N_2 at 22° and 747 mm. 0.209 gm. histidine dichloride. 104.5%
Coli P–5–19.	0.10 cc. = 9.8 mm. 0.20 " = 19.4 " Match perfect. 98%	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. 79%	1.77 cc. N_2 at 20° and 745 mm. 0.201 gm. histidine dichloride. 100.5%
Coli P–6–19.	0.10 cc. = 9.4 mm. 0.20 " = 18.9 " Match perfect. 94%	0.10 cc. = 9.4 mm. 0.20 " = 18.8 " Match perfect. 94%	1.81 cc. N_2 at 21° and 746 mm. 0.205 gm. histidine dichloride. 102.5%

* Colors matched against the (CR–MO) standard.

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Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.				7.4	5.8
None.			38 Hence the = of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			31 Hence the = of 5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.7
None.			15.5 Hence the = of 20.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.7
None.			32.5 Hence the = of 3.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	5.9
0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Color develops like that of histamine.	0.0235 of histamine dichloride in the entire test solution. 14.5% of histamine present.	0.35 cc. N_2 at 22° and 744 mm. 0.0317 gm. histamine dichloride. 19.6%	35.5 Hence the = of 0.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	6.2
None.			27 Hence the = of 9 cc. of 0.1 N NH_3 was used by the microorganisms.	7.4	6.0

TABLE VI—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Coli I (K).	0.10 cc. = 9.0 mm. 0.20 " = 18.1 " Match perfect. 90%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.71 cc. N_2 at 22.5° and 745 mm. 0.1925 gm. histidine dichloride. 96.2%
Coli Schwartz.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. 89%	1.89 cc. N_2 at 28° and 749 mm. 0.207 gm. histidine dichloride. 103.5%
Coli 52.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.87 cc. N_2 at 27° and 746 mm. 0.205 gm. histidine dichloride. 102.5%
Coli 51 (white).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	1.89 cc. N_2 at 28° and 749 mm. 0.207 gm. histidine dichloride. 103.5%
Coli 74.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.8 mm. 0.20 " = 17.6 " Match perfect. 88%	1.83 cc. N_2 at 28° and 747 mm. 0.200 gm. histidine dichloride. 100%

Concluded.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.			32.5 Hence the ∞ of 3.5 cc. of 0.1 N NH_3 was used by the microorganisms.	7.3	5.5
None.			38.5 Hence the ∞ of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.6
None.			39 Hence the ∞ of 3 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.4
None.			38 Hence the ∞ of 2 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	7.0
None.			37 Hence the ∞ of 1 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.4	5.5

TABLE VII—*Colon*

Name of strain.	Total color value of test solution of histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus enteritidis 228, 7 days.	0.10 cc. = 8.6 mm. 0.20 " = 17.1 " Match perfect. 86%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.46 cc. N_2 at 29° and 746 mm. 0.1584 gm. histidine dichloride. 79.2%
14 days.	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match poor. Color too yellow. 40%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	1.25 cc. N_2 at 23° and 745 mm. 0.140 gm. histidine dichloride. 70%
Bacillus typhosus.	0.10 cc. = 9.6 mm. 0.20 " = 19.1 " Match perfect. 96%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.35 cc. N_2 at 21° and 743 mm. 0.152 gm. histidine dichloride. 76%
Bacillus paratyphosus A 3.	0.10 cc. = 9.3 mm. 0.20 " = 18.6 " Match perfect. 93%	0.10 cc. = 8.3 mm. 0.20 " = 16.7 " Match perfect. 83%	1.47 cc. N_2 at 20° and 738 mm. 0.1655 gm. histidine dichloride. 82.7%
Bacillus paratyphosus A 4, 7 days.	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	1.73 cc. N_2 at 28° and 746 mm. 0.1886 gm. histidine dichloride. 94.3%
14 days.	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match good. 40%	1.95 cc. N_2 at 24° and 753 mm. 0.2195 gm. histidine dichloride. 109.7%
Bacillus paratyphosus A (K).	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine. 100%	0.95 cc. N_2 at 24° and 753 mm. 0.107 gm. histidine dichloride. 53.5%

* Colors matched against the (CR-MO) standard.

Typhoid Group.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	24.5 Hence the = of 11.5 cc. of 0.1 N NH_3 used by the microorganisms.	7.3	6.0
None.	None.	None.	39.5 Hence the = of 15 cc. of 0.1 N NH_3 was produced during the second 7 day period.	7.3	5.2
	None.	None.	29 Hence the = of 7 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	6.6
None.	None.	None.	31.5 Hence the = of 4.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
None.	None.	None.	39 Hence the = of 3 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	6.4
None.	None.	None.	38 Hence the = of 1 cc. of 0.1 N NH_3 was removed during the second 7 day period.	7.3	5.4
0.034 gm. 27.7%	None	None.	30.5 Hence the = of 5.5 cc. of 0.10 N NH_3 was removed by the microorganisms.	7.3	6.8

TABLE VII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus dysenteriæ Flexner.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too pink and develops too rapidly for histamine. 100%	0.10 cc. = 9.6 mm. 0.20 " = 19.2 " Color too pink and develops too rapidly for histamine. 96%	0.91 cc. N_2 at 22° and 745 mm. 0.1025 gm. histidine dichloride. 51.2%
Bacillus dysenteriæ Morgan.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect, color develops rapidly. 95%	0.10 cc. = 7.8 mm. 0.20 " = 15.6 " Match perfect, color develops rapidly. 78%	1.15 cc. N_2 at 22° and 743 mm. 0.129 gm. histidine dichloride. 65%
Bacillus dysenteriæ Shiga.	0.10 cc. = 11.3 mm. 0.20 " = 22.6 " Color too pink and develops too rapidly for histamine. 113%	0.10 cc. = 9.3 mm. 0.20 " = 18.5 " Color too pink and develops too rapidly for histamine. 93%	1.24 cc. N_2 at 19° and 748 mm. 0.1423 gm. histidine dichloride. 71.1%
Bacillus fæcalis alcaligenes I.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Color slightly too pink. 97%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Color slightly too pink for histidine. 70%	0.80 cc. N_2 at 23° and 749 mm. 0.090 gm. histidine dichloride. 45%
Bacillus fæcalis alcaligenes III, 3 days.	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match good. 90%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Match perfect. 70%	1.30 cc. N_2 at 23° and 750 mm. 0.1464 gm. histidine dichloride. 73.2%
14 days.	0.10 cc. = 9.2 mm. 0.20 " = 18.5 " Match perfect. 92%	0.10 cc. = 7.0 mm. 0.20 " = 14.0 " Match perfect. 70%	1.17 cc. N_2 at 23° and 749 mm. 0.1317 gm. histidine dichloride. 66%

Concluded.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
0.033 gm. 27.1%	None.	None.	34.5 Hence the = of 1.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.010 gm. 8.1%	None.	None.	35.5 Hence the = of 0.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.016 gm. 13%	None.	None.	33 Hence the = of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.018 gm. 14.65%	None.	None.	32.5 Hence the = of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4
None.	None.	None.	32.5 Hence the = of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4
None.	None.	None.	33 Hence the = of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.6

B. paratyphosus A 3.—This organism grew very poorly on our medium, apparently because an available carbon source was lacking. Both the glycerol and the histidine remained largely unattacked. Histamine, other imidazoles, and acid were not formed. Of the ammonia originally introduced, 13 per cent was removed by the bacilli.

B. paratyphosus A 4.—This organism grew far better than *B. paratyphosus* A 3. Some of the glycerol was metabolized with the production of acid. The imidazole ring was ruptured to a considerable extent with the formation of carboxylated amino compounds and ammonia. Histamine and other imidazoles were not formed.

B. paratyphosus A (K).—This organism deaminized histidine with the formation of imidazole acetic, propionic, lactic, or acrylic acids because the color produced by the histidine fraction was far too red for histidine and the amino nitrogen value was much lower than the colorimetric value. If we assume that *the amino nitrogen was derived exclusively from histidine*, the presence of a maximum of 53.5 per cent of that substance is indicated. Colorimetrically this would give a reading of 5.35 mm. (CR − MO) for 0.10 cc. of the diluted test solution. The reading obtained was 10.0 mm. (CR − MO) for 0.10 cc. The difference between these two readings—4.65 mm. (CR − MO) or 5.12 mm. (CR)[8]—must have been due to either imidazole acetic, propionic, lactic, or acrylic acids. Which of these acids was present was not determined; but the excess color value was calculated as imidazole propionic acid using the table that was previously published for that substance. The calculations show that 0.034 gm. of imidazole propionic acid was present; hence 27.7 per cent of the histidine originally introduced was deaminized.

This organism grew poorly, used glycerol to only a small extent, produced very little acid, and removed the equivalent of 16 per cent of the ammonia originally introduced. Histamine was not formed.

B. dysenteriæ Flexner, Morgan, and Shiga.—The three classes of dysentery bacilli can most advantageously be discussed together because, although the results are quantitatively somewhat different, they are qualitatively identical. In every case the color obtained with the histidine fraction was too red to have been due only to histidine. The colorimetric values were higher than the amino nitrogen values on this fraction; hence histidine was not the only imidazole present. When the discrepancies between the values obtained colorimetrically and by the amino nitrogen method are calculated as imidazole propionic acid, the presence of 0.033 gm. (27.1 per cent), 0.01 gm. (8.1 per cent), and 0.016 gm. (13 per cent) of this substance is indicated for Flexner, Morgan, and Shiga respectively.

These organisms grew poorly, used glycerol only to a small extent, produced practically no acid, and removed very little ammonia from the solution. Histamine was not formed.

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[8] The (CR) standard is $\frac{1}{10}$ as intense as the (CR − MO) standard.

B. fæcalis alcaligenes I.—The color obtained with the histidine fraction was too red for histidine and the amino nitrogen value was 25 per cent lower than the colorimetric value. This suggests the presence of imidazole acetic, propionic, lactic, or acrylic acids. When the discrepancy between the values obtained colorimetrically and by the amino nitrogen method are calculated as imidazole propionic acid, the presence of 0.018 gm. (14.65 per cent) of this substance is indicated.

This organism grew so poorly that the medium was almost clear after 14 days of incubation. Some of the bacilli were, nevertheless, alive at the end of this period. Little or no use was made of the glycerol, and acid was not produced. Of the ammonia originally introduced, 90 per cent was recovered. Histamine was not formed.

B. fæcalis alcaligenes III.—This organism grew about as poorly as *B. fæcalis alcaligenes* I. During the first 3 days, 30 per cent of the histidine was removed. After that there was practically no change in the histidine content of the liquid. Imidazole propionic acid and histamine were not formed. Of the ammonia originally introduced, 90 per cent was recovered. The slight production of alkalinity during the las 11 days of incubation may have been caused by autolytic changes because apparently the active life of the organisms came to an end after 3 days of meager growth.

PART III.

On the Products Formed from Histidine by the Action of Organisms Other than Those Belonging to the Colon Typhoid Groups.

The organisms investigated were *Bacillus mucosus capsulatus* (2 strains), *Bacillus bifidus*, *Bacillus influenzæ*, *Bacillus proteus vulgaris* (2 strains), *Bacillus cloacæ*, *Streptococcus hæmolyticus*, (2 strains), Pneumococcus Types I, II, III, and IV, and *Bacillus tuberculosis* (5 strains). The behavior of these organisms on our synthetic medium is summarized in Table VIII.

B. mucosus capsulatus.—An excellent growth was obtained. This organism attacked the histidine immediately and so effectively that 75 per cent of it was destroyed in 14 days. The histidine seems to have been attacked because of its nitrogen. The following facts led us to this conclusion:

1. The ammonia concentration remained practically unchanged throughout the entire course of the experiment in spite of the fact that an unusually abundant growth was obtained. The nitrogen requirements of the organism must, therefore, have been obtained from the histidine.

2. Although the histidine was largely removed from the solution, none of its nitrogen appeared as ammonia and only a very small amount of it appeared in the amino condition. During the first few days, all of the nitrogen derived from the disrupted histidine was consumed. Toward the

TABLE VIII—*Miscel*

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus mucosus capsulatus L. S., 3 days.	0.10 cc. = 8.2 mm. 0.20 " = 16.4 " Match perfect. 82%	0.10 cc. = 7.3 mm. 0.20 " = 14.5 " Match perfect. 73%	1.33 cc. N_2 at 18° and 750 mm. 0.1535 gm. histidine dichloride. 76.8%
14 days.	0.10 cc. = 2.8 mm. 0.20 " = 5.6 " Match fair. 28%	0.20 cc. = 5.1 mm. 0.40 " = 10.2 " Match good. 25.5%	0.75 cc. N_2 at 25° and 745 mm. 0.083 gm. histidine dichloride. 41.5%
Bacillus mucosus capsulatus L. S. plus 0.10 gm. leucine, 14 days.	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match good. 80%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match good. 75%	1.46 cc. N_2 at 21° and 742 mm. 0.1655 gm. histidine dichloride. 82.7%
Bacillus mucosus capsulatus 27 (S), 3 days.	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. 75%	0.46 cc. N_2 at 23° and 752 mm. 0.052 gm. histidine dichloride. 26%
5 days.	0.20 cc. = 5.8 mm. 0.40 " = 11.6 " Match good. 29%	0.20 cc. = 4.9 mm. 0.40 " = 9.8 " Match good. 23%	0.80 cc. N_2 at 21° and 737 mm. 0.0894 gm. histidine dichloride. 44.7%
8 days.	0.50 cc. = 2.4 mm. Color brownish yellow. Match poor. 4.8%	0.50 cc. = 2.0 mm. 1.00 " = 4.0 " Match good. 4.0%	0.75 cc. N_2 at 21° and 737 mm. 0.0838 gm. histidine dichloride. 41.9%
Bacillus bifidus, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.7 mm. 0.20 " = 17.4 " Match perfect. 87%	1.55 cc. N_2 at 23° and 744 mm. 0.173 gm. histidine dichloride. 86.5%

*Colors matched against the (CR–MO) standard.

laneus Organisms.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	36.7 Hence the = of 0.7 cc. of 0.1 N NH_3 was produced.	7.3	6.2
None.	None.	None.	36.7 An ammonia equilibrium was established after 7 days of growth.	7.3	6.8
None.	None.	None.	34 Hence the = of 2 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.4
0.0365 gm. 29.7%	None.	None.	38.5 Hence the = of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.2
None.	None.	None.	44 Hence the = of 8 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.1
None.	None.	None.	49 Hence the = of 13 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.0
None.	None.	None.	32.5 Hence the = of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Bacillus influenzæ, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Match perfect. 90%	1.55 cc. N_2 at 32° and 746 mm. 0.165 gm. histidine dichloride. 82.5%
Bacillus proteus vulgaris 186, 7 days.	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match good. 76%	0.10 cc. = 6.9 mm. 0.20 " = 13.7 " Match good. 69%	1.13 cc. N_2 at 21° and 754 mm. 0.1294 gm. histidine dichloride. 64.7%
14 days.	0.20 cc. = 9.0 mm. 0.30 " = 13.7 " Color too yellow for histidine. 45%	0.20 cc. = 9.0 mm. 0.30 " = 13.5 " Match fair. Color slightly too yellow. 45%	0.78 cc. N_2 at 21° and 752 mm. 0.089 gm. histidine dichloride. 44.5%
Bacillus proteus vulgaris A. I. K., 14 days.	0.10 cc. = 8.1 mm. 0.20 " = 16.2 " Match perfect. 81%	0.10 cc. = 7.4 mm. 0.20 " = 14.7 " Match perfect. 74%	2.20 cc. N_2 at 20° and 738 mm. 0.2475 gm. histidine dichloride. 123.7%
Bacillus cloacæ I, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	1.85 cc. N_2 at 26° and 745 mm. 0.2036 gm. histidine dichloride. 101.8%
Streptococcus hæmolyticus 4A, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.51 cc. N_2 at 22° and 748 mm. 0.1705 gm. histidine dichloride. 85.2%
Streptococcus hæmolyticus II R, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.65 cc. N_2 at 28° and 746 mm. 0.180 gm. histidine dichloride. 90%

Continued.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
	None.	None.	31.5 Hence the = of 4.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.3
None.	None.	None.	35 Hence the = of 1 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
None.	None.	None.	40 Hence the = of 4 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.1
None.	None.	None.	28 Hence the = of 8 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.2
None.	None.	None.	30 Hence the = of 6 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.5
None.	None.	None.	32.5 Hence the = of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.3
None.	None.	None.	33 Hence the = of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.4

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Pneumococcus Type I, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.4 mm. 0.20 " = 16.8 " Match perfect. 84%	1.48 cc. N_2 at 21° and 750 mm. 0.1684 gm. histidine dichloride. 84.2%
Pneumococcus Type I plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. 86%	1.54 cc. N_2 at 29° and 746 mm. 0.167 gm. histidine dichloride. 84%
Pneumococcus Type II, 14 days.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	0.10 cc. = 7.8 mm. 0.20 " = 15.5 " Match perfect. 78%	1.38 cc. N_2 at 32° and 748 mm. 0.1475 gm. histidine dichloride. 74%
Pneumococcus Type II plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. 89%	2.05 cc. N_2 at 32° and 746 mm. 0.2185 gm. histidine dichloride. 109.2%
Penumococcus Type III, 14 days.	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Match perfect. 95%	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. 79%	1.65 cc. N_2 at 32° and 748 mm. 0.1765 gm. histidine dichloride. 88.2%
Pneumococcus Type III plus 0.10 gm. leucine, 14 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. 100%	0.10 cc. = 8.5 mm. 0.20 " = 17.0 " Match perfect. 85%	1.81 cc. N_2 at 32° and 748 mm. 0.1935 gm. histidine dichloride. 96.8%
Pneumococcus Type IV, 14 days.	0.10 cc. = 9.7 mm. 0.20 " = 19.3 " Match perfect. 97%	0.10 cc. = 8.0 mm. 0.20 " = 16.0 " Match perfect. 80%	1.47 cc. N_2 at 31° and 748 mm. 0.158 gm. histidine dichloride. 79%

Continued.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	33 Hence the = of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
None.	None.	None.	33 Hence the = of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	33 Hence the = of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the = of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the = of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
None.	None.	None.	32 Hence the = of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
None.	None.	None.	33 Hence the = of 3 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1

TABLE VIII—

Name of strain.	Total color value of test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Pneumococcus Type IV plus 0.10 gm. leucine, 14 days.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Match perfect. 97%	0.10 cc. = 7.9 mm. 0.20 " = 16.0 " Match perfect. 79%	1.60 cc. N_2 at 31° and 748 mm. 0.172 gm. histidine dichloride. 86%
Bacillus tuberculosis H. Sp., 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.0 mm. 0.20 " = 18.0 " Color too red for histidine. 90%	1.23 cc. N_2 at 22° and 756 mm. 0.140 gm. histidine dichloride. 70%
Bacillus tuberculosis 1305, 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Color too red for histidine. 95%	0.95 cc. N_2 at 20° and 750 mm. 0.1085 gm. histidine dichloride. 54.2%
Bacillus tuberculosis 3161, 45 days.	0.10 cc. = 9.6 mm. 0.20 " = 19.3 " Color too red for histidine. 96%	0.10 cc. = 8.1 mm. 0.20 " = 16.2 " Color too red for histidine. 81%	0.95 cc. N_2 at 20° and 752 mm. 0.109 gm. histidine dichloride. 54.5%
Bacillus tuberculosis O. H., 45 days.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Color too red for histidine. 100%	0.10 cc. = 9.5 mm. 0.20 " = 19.0 " Color too red for histidine. 95%	1.09 cc. N_2 at 21° and 761 mm. 0.126 gm. histidine dichloride. 63%
Bacillus tuberculosis (Novy) K_1, 5 days.	0.10 cc. = 5.8 mm. 0.20 " = 11.5 " Match fair. Color too yellow. 58%	0.10 cc. = 4.7 mm. 0.20 " = 9.2 " Match fair. Color too yellow. 47%	1.27 cc. N_2 at 18° and 740 mm. 0.1446 gm. histidine dichloride. 72.3%
45 days.	1.00 cc. = 15.0 mm. Color brown. Match poor. 15%	1.00 cc. = 12.0 mm. Color brown. Match poor. 12%	0.81 cc. N_2 at 21° and 762 mm. 0.0936 gm. histidine dichloride. 46.8%

Concluded.

Histidine converted into imidazole propionic acid (by difference).	Color value of histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.1 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
				Before incubation.	After incubation.
			cc.	*pH*	*pH*
None.	None.	None.	32 Hence the = of 4 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.015 gm. 12.2%	None.	None.	27 Hence the = of 9 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.0
0.030 gm. 24.5%	None.	None.	28.5 Hence the = of 7.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.1
0.020 gm. 16.3%	None.	None.	31 Hence the = of 5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	7.2
0.023 gm. 18.7%	None.	None.	32.5 Hence the = of 3.5 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	6.9
None.	None.	None.	38.5 Hence the = of 2.5 cc. of 0.1 N NH_3 was produced by the microorganisms.	7.3	7.2
None.	None.	None.	37.5 Hence the = of 1 cc. of 0.1 N NH_3 was removed during the 40 day period.	7.3	6.8

end of the 2 week period non-volatile amino nitrogen was liberated slightly faster than it was being consumed.

3. When leucine was added to the medium, along with the histidine, the growth of the organisms was augmented; but it was the leucine nitrogen that was removed from the solution, the histidine being largely unattacked. That the leucine nitrogen was removed is proved by the fact that the amino nitrogen value on the histidine fraction ran only slightly higher than the colorimetric value for histidine.

One might be led to assume that this organism cannot use ammonia to satisfy its nitrogen requirements. This is, however, not the case because a very good growth is obtained in a medium containing only glycerol and ammonium chloride together with the usual inorganic salts. In this case considerable ammonia is removed by the microorganisms. It seems that this organism used amino nitrogen in preference to ammonia nitrogen. Histamine and other imidazoles were not formed.

In addition to the above stock culture, two strains of *Bacillus mucosus capsulatus* were isolated in this laboratory, from the feces of two cases of colitis. They were quantitatively almost identical in their action. The horizontal Columns 4, 5, and 6 contain a summary of the behavior of one of them.

An excellent growth was obtained. The amino nitrogen value of the histidine fraction, obtained after 3 days of incubation, shows that a maximum of only 26 per cent of unchanged histidine could have been present. The color obtained with this fraction was too red to have been exclusively due to histidine, and the color value was far too high to agree with the amino nitrogen value; hence the presence of imidazole acetic, propionic, lactic, or acrylic acids is indicated. This discrepancy between the color value and the amino nitrogen value is equal to 0.0365 gm. (29.7 per cent) calculated as imidazole propionic acid. This organism therefore attacks the histidine in two distinct ways to obtain nitrogen. Some of the molecules are deaminized, others suffer a nuclear disruption. Some, but not much, ammonia is generated during the first 3 days.

During the next 2 days the imidazole propionic (?) acid formed at first was destroyed with the liberation of some non-volatile primary amine and considerable ammonia. The color obtained with the histidine fraction in this case was just like that obtained with histidine. We have assumed, therefore, that the color was at least *largely* due to histidine. If this conclusion is correct, the attack during this 2 day period was directed almost exclusively against the imidazole propionic acid formed during the first 3 days.

During the next 2 days the remaining histidine was destroyed almost completely with the liberation of ammonia. Histamine was not formed at any time.

The three strains of *Bacillus mucosus capsulatus* studied by us satisfied their nitrogen requirements at the expense of the amino-acid histidine in preference to ammonia. Two of the strains deaminized histidine and ruptured the nucleus simultaneously. The other strain merely ruptured the nucleus. Glycerol was, in every case, the chief source of carbon.

B. bifidus.—This organism grew very poorly on our medium, apparently because an available carbon source was lacking. The glycerol originally introduced seemed not to have been attacked. Of the histidine originally introduced, 87 per cent was recovered. There is no evidence that the nucleus was ruptured. The equivalent of 90 per cent of the initial ammonia was recovered. Histamine was not formed. When leucine was added to the medium the organisms grew for a few days during which time *all* the leucine nitrogen disappeared and some acid was produced. Histamine was not produced in this case.

B. influenzæ.—This organism grew very poorly on our medium. There is slight evidence for the formation of imidazole propionic (etc.) acids. Of the ammonia originally introduced, 91 per cent was recovered. Acid was not produced; glycerol was not utilized.

When leucine (0.10 gm.) was added to the medium, the organisms grew for a few days. They apparently found, in leucine, an easily available source of carbon and nitrogen because none of the leucine nitrogen could be accounted for at the end of 14 days. In this case 95 per cent of the histidine originally introduced was recovered. Histamine was not formed in any case.

B. proteus vulgaris 186.—This organism grew poorly on our medium apparently because an easily available source of carbon was lacking. Acid was not produced; glycerol was not attacked. The histidine was progressively destroyed, the excess nitrogen appearing as ammonia. The organisms must, therefore, have destroyed the histidine to obtain carbon. Histamine and other imidazoles were not formed.

B. proteus vulgaris A. I. K.—This organism grew very well on our medium. Glycerol was consumed and acids were produced. The great discrepancy between the color and amino nitrogen values for histidine is good evidence that a carboxylated amino compound was produced from the histidine. Since this organism was able to use ammonia as a source of nitrogen, the excessive amine production could hardly have been resorted to because of the nitrogen thus rendered available. The amines were more probably produced to lower the hydrogen ion concentration of the cell protoplasm. The equivalent of 77 per cent of the ammonia originally introduced was recovered. Histamine and other imidazoles were not formed.

B. cloacæ I.—As far as we could tell, this organism died shortly after it was introduced into our medium. The histidine and the glycerol were not attacked.

Streptococcus hæmolyticus 4.1 and II R.—Neither of these organisms grew perceptibly on our medium although they were still alive at the end of 14 days. Glycerol was not consumed; acid was not produced. About 85 per cent of the histidine originally introduced was recovered in each case. The initial ammonia concentration was reduced by only 10 per cent. Histamine and other imidazoles were not formed.

The results obtained with a strain of *Streptococcus viridans* were quantitatively almost identical with those obtained above.

Pneumococci, Types I, II, III, and IV.—These organisms grew poorly on our medium. They removed 15 to 20 per cent of the histidine originally introduced in such a way that the nitrogen cannot be accounted for either as NH_3 or as NH_2. There is no evidence that a nuclear rupture occurred. The constancy of the ammonia value indicates strongly that these organisms do not use it as a source of nitrogen. Histamine and other imidazoles were not formed.

When leucine (0.10 gm.) was added to the medium the organisms multiplied rapidly for a few days. The addition of leucine had no effect upon the NH_3 and pH values and the histidine was removed to about the same extent as in the leucine-free medium. The leucine, however, was almost completely destroyed in every case. This would seem to indicate that leucine is a good source of nitrogen and carbon for the pneumococcus while histidine, ammonia, and glycerol are poor sources of these elements.

B. tuberculosis H.Sp., 1305, 3161, and O.H.—These four strains of tubercle bacilli gave results that were qualitatively identical though quantitatively somewhat different. In every case there is good evidence that imidazole acetic, propionic, lactic, or acrylic acids were present. The color obtained with the histidine fraction was too red for histidine. The values obtained for histidine by the amino nitrogen method were always decidedly lower than those obtained colorimetrically. The discrepancies between the color values and the amino nitrogen values are equal to 0.015 gm. (12.2 per cent) for H.Sp., 0.030 gm. (24.5 per cent) for No. 1305, 0.02 gm. (16.3 per cent) for No. 3161, and 0.023 gm. (18.7 per cent) for O.H., calculated as imidazole propionic acid. The organisms grew very well on the surface of our medium. An excess of acid was not produced during this time interval although the glycerol had been largely destroyed. The ammonia consumption was distinctly different in the four cases, varying from 40 per cent in the case of H.Sp. to only 10 per cent in the case of O.H. Histamine was not produced in any case.

A striking contrast to these four organisms was that of a fifth strain which is widely known as K_1. This organism has been grown on synthetic media for so long—about 40 years—that it has lost its virulence entirely. It grows excellently on our medium, the growth not being confined to the surface. Chemically it did not behave like a typical tubercle bacillus as can be seen by examining the last two horizontal columns of Table VIII. This organism attacked the histidine immediately and so effectively that 53 per cent of it had been destroyed at the end of 5 days of incubation. Some of the histidine nitrogen was converted into ammonia; some appeared as a non-volatile, carboxylated amine. After 45 days of incubation only 12 per cent of the histidine originally introduced remained. An excess of acid was not formed although the glycerol had been destroyed. Imidazole propionic acid and histamine were not produced.

In concluding this part of the work we wish to call special attention to the fact that these experiments have all been carried out in an artificial synthetic medium. Our conditions are, at

best, highly artificial and the composition of our synthetic medium is far simpler than that of living tissue or even than that of the usual composite culture media. We are well aware that results obtained in such a medium must be applied carefully, if at all, to the general problem of amine production in the living organism. We feel, however, that our mode of procedure is justified by the fact that we have first to develop methods for estimating imidazoles and phenols under simple conditions before we can hope to estimate these substances in complex mixtures. The final answer to the question, "which microorganisms are probable amine producers in the human organism" can only be given after a medium has been used that contains all of the essential ingredients of a tissue or tissue extract. How definitely the amine production is influenced by the composition of the medium is shown clearly in the Part IV of this report where other amino-acids or peptones were added to our synthetic medium.

PART IV.

The Production of Histamine by Bacillus coli cystitis when Other Amino-Acids Are Present in the Medium Together with Histidine.

This investigation was undertaken to ascertain which amino-acids when added to our medium, augment the production of histamine and the growth of the microorganisms. The colon bacillus used in this work (*Bacillus coli cystitis*) was the strain employed by us in our original investigation.[1] We have found that this organism will always convert approximately 50 per cent of the histidine originally introduced into histamine when precautions are taken to have the initial pH of the medium 7.3 and to maintain a uniform temperature of 37°.

In the first experiment of this series we compared the carboxylase activity on our standard medium with that on a medium containing leucine and histidine in one case, and peptone and histidine in the other. The results obtained are summarized in Table IX.

The results obtained in the medium containing histidine as the only amino-acid call for very little special comment because they closely resemble those obtained and reported 2 years ago. In

TABLE IX—*Effect of Leucine and Peptone on the*

Composition of the medium.	Total color value of the test solution as histidine dichloride.* (0.20 gm. = 100%.)	Color value of histidine fraction as histidine dichloride.* (0.20 gm. = 100%.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.
Histidine dichloride, 0.2 gm. Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Match perfect. Color develops promptly. 112%	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. 40%	0.85 cc. N_2 at 20° and 750 mm. 0.097 gm. histidine dichloride. 48.5%
Histidine dichloride, 0.2 gm. Leucine, 0.1 " Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 10.3 mm. 0.20 " = 20.6 " Match perfect. Color develops promptly. 103%	0.50 cc. = 4.5 mm. 1.00 " = 9.0 " Match poor. Color too yellow. 9.0%	0.72 cc. N_2 at 21° and 753 mm. 0.0822 gm. histidine dichloride. 41.1%
Histidine dichloride, 0.2 gm. Peptone (Witte), 0.05 " Potassium nitrate, 0.1 " Ammonium chloride, 0.2 " Glycerol, 4.0 cc. Other inorganic salts as per Nutritive Medium 3. Distilled water to 200 cc.	0.10 cc. = 11.2 mm. 0.20 " = 22.4 " Match perfect. Color develops promptly. 112%	0.20 cc. = 3.0 mm. 0.40 " = 6.0 " Match good. 15%	0.65 cc. N_2 at 22° and 754 mm. 0.074 gm. histidine dichloride. 37%

*Colors matched against the (CR–MO) standard.

Producion of Histamine by Bacillus coli (cystitis).

Color value of the histamine fraction.*	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	0.10 N HCl neutralized by NH_3 from entire test solution.	Reaction.	
			Before incubation.	After incubation.
		cc.	*pH*	*pH*
0.05 cc. = 13.8 mm. 0.10 " = 27.5 " Color developed like that of histamine. 0.092 gm. of histamine dichloride in entire test solution. 57% of histidine converted into histamine.	1.04 cc. N_2 at 20° and 754 mm. 0.0964 gm. histamine dichloride. 59.8%	27 Hence the = of 9 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.4
Color too intense to run directly; hence 10 cc. were diluted to 100 cc. Of this solution 0.20 cc. = 7.6 mm. 0.40 " = 15.2 " Color develops like that of histatamine. 0.1275 gm. of histamine dichloride in entire test solution. 79% of histidine converted into histamine.	2.55 cc. N_2 at 22° and 744 mm. 0.231 gm. histamine dichloride. 143%	25 Hence the = of 11 cc. of 0.1 N NH_3 was removed by the microorganisms.	7.3	5.5
Color too intense to run directly; hence 10 cc. were diluted to 100 cc. Of this solution 0.25 cc. = 9.3 mm. 0.50 " = 18.5 " Color develops like that of histamine. 0.125 gm. of histamine dichloride in entire test solution. 77.4% of histidine converted into histamine.	1.45 cc. N_2 at 23° and 755 mm. 0.133 gm. histamine dichloride. 82.4%		7.3	5.2

this case 57 per cent of the histidine originally introduced was converted into histamine. The agreement between the values obtained colorimetrically and by the amino nitrogen method is good.

Although the organisms multiplied rapidly in the above medium, they grew far better in a medium containing leucine or peptone together with the histidine. When leucine was present, 79 per cent of the histidine originally introduced was converted into histamine, as determined from the colorimetric reading an increase of 22 per cent over the leucine-free medium. The amino nitrogen value is obviously much too high to represent histamine alone. A portion of the leucine may have been converted into isoamylamine which would appear together with histamine in the amyl alcohol extract and thus raise the amino nitrogen value of the histamine fraction.

In the presence of peptone, 77 per cent of the histidine originally introduced was converted into histamine; hence leucine and peptone are about equivalent in their ability to promote histamine formation. In this case the check between the colorimetric and amino nitrogen values is sufficiently close to warrant the conclusion that other amines were not formed in appreciable quantities. In this case the acidity was also neutralized less perfectly than in the case of leucine where a large amount of amine production, other than histamine, was indicated.

In short then, we would conclude that the presence of either leucine or peptone stimulated the production of histamine. We can draw no conclusions from this experiment, as to the rôle played by the leucine or peptone in the amine production. We might conclude, with Jacoby,[9] that leucine was an easily available source from which carboxylase enzymes could be synthesized; but our experiment seems to indicate that the chief factor is the enormous increase in the speed of growth of the microorganisms which would, of course, increase the rate of acid production and hence render the early formation of histamine necessary to neutralize the acid.

We have grown *all* of the organisms discussed in the first three sections of this paper on a medium containing both leucine and histidine and have found that:

[9] Jacoby, M., *Biochem. Z.*, 1917, lxxxi, 332; lxxxiii, 74; lxxxiv, 358; 1918, lxxxvi, 329.

1. The addition of leucine always facilitates the growth of the organisms.

2. If the organisms produce no histamine in the absence of leucine, they will not produce histamine when leucine is present.

3. If the organisms produce histamine when leucine is absent, they will always produce 20 to 25 per cent more of this amine, in 2 weeks of incubation, when leucine is present. The leucine augments a power that already exists; it does not call forth a new enzymatic activity.

The second series was an enlargement upon the first for in this case all of the easily available amino-acids were employed; namely, glycine, alanine, cystine, leucine, arginine, glutamic acid, tyrosine, and tryptophane. In addition to the above amino-acids two flasks were prepared containing different makes of peptone; namely, Witte and Difco. The results obtained are summarized in Table X.

Table X shows that the histamine value on our standard medium was 20 per cent lower this time than it has been in any of our other experiments with this organism. The value on the leucine-histidine medium is also 20 per cent low. All of the flasks of *this* series were incubated at the same time in the same incubator. During the first few days the air in the incubator smelled strongly of hydrogen sulfide which was being evolved from the cystine-containing flask. We feel convinced that the retardation in speed of activity, and probably in rate of growth, was caused by the hydrogen sulfide. This conclusion is strengthened by the fact that only 2 per cent of histamine was formed in the cystine-histidine medium. The growth in this medium was also very meager. Since this hydrogen sulfide interference must have been approximately equivalent for all of the media, we believe that the values obtained are accurate in so far as they may be compared with one another. In discussing this series, a conversion of 31 per cent of the histidine originally introduced into histamine is considered to be the normal conversion on our standard medium. When more than 31 per cent of histamine was present, the amino-acid added can be considered to be an amine production *stimulator*, and *vice versa*. The amino nitrogen values on both the histidine and histamine fractions have, in every case, been calculated as histidine and as histamine, respectively, in spite of the fact

TABLE X—*Effect of Amino-Acids and Peptones on*

Composition of the medium.	Histidine dichloride, 0.2 gm. KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Glycine, 0.065 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Alanine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Leucine, 0.115 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Cystine, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
Total color value calculated as % of histidine dichloride. (0.20 gm. = 100%), *per cent.*	112	108	100	112	96
Unchanged histidine colorimetric method. (0.20 gm. = 100%), *per cent.*	60	43	30	37	87
Amino nitrogen value of histidine fraction calculated as % of histidine dichloride. (0.20 gm. = 100%), *per cent.*	74	102.5	154	55	147
Histidine converted into histamine (colorimetric method) *per cent.*	31	37.2	51	49.6	2.06
Amino nitrogen value of histamine fraction calculated as % of histamine dichloride, *per cent.*	32	49	70.7	152	

the Production of Histamine by Bacillus coli (cystitis.)

Histidine dichloride, 0.2 gm. Glutamic acid HCl, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Arginine carbonate, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tyrosine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tryptophane, 0.18 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Witte), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Difco), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
105	110		99	103	97
62	52	11.5	60	40	50
115.5	109.8	10.6	79.5	60.6	67.1
21.7	41	31.4	26.3	42	42
42.5	108.4			56.6	62.8

TABLE X—

Composition of the medium.	Histidine dichloride, 0.2 gm. KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Glycine, 0.065 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts Distilled water to 200 "	Histidine dichloride, 0.2 gm. Alanine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Leucine, 0.115 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Cystine, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
0.10 N HCl neutralized by NH_3 from the entire test solution, *cc.*	20.5 Hence the ⇔ of 16 cc. of 0.1 N NH_3 was used by microorganisms.	16.5 Hence the ⇔ of 19.5 cc. of 0.1 N NH_3 was used by microorganisms.	17 Hence the ⇔ of 19 cc. of 0.1 N NH_3 was used by microorganisms.	23.7 Hence the ⇔ of 12.3 cc. of 0.1 N NH_3 was used by microorganisms.	9.2 Hence the ⇔ of 26.8 cc. of 0.1 N NH_3 was used by microorganisms.
pH after 14 days of incubation. The initial pH was 7.3 in all cases, *pH.*	5.5	5.5	5.6	5.9	5.3
Tyrosine converted into volatile phenols (colorimetric method), *per cent.*					
Tyrosine converted into aromatic hydroxy-acids. Calculated as oxyphenyllactic acid (colorimetric method) *per cent.*					
Unchanged tyrosine (colorimetric method), *per cent.*					
Tyrosine converted into tyramine (colorimetric method) *per cent.*					

Concluded.

Histidine dichloride, 0.2 gm. Glutamic acid HCl, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Arginine carbonate, 0.2 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tyrosine, 0.16 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Tryptophane, 0.18 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Witte), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "	Histidine dichloride, 0.2 gm. Peptone (Difco), 0.05 " KNO_3, 0.1 " NH_4Cl, 0.2 " Glycerol, 4.0 cc. Usual inorganic salts. Distilled water to 200 "
17.5 Hence the ∞ of 18.5 cc. of 0.1 N NH_3 was used by microorganisms.	19.5 Hence the ∞ of 16.5 cc. of 0.1 N NH_3 was used by microorganisms.	23.5 Hence the ∞ of 12.5 cc. of 0.1 N NH_3 was used by microorganisms.	20 Hence the ∞ of 16 cc. of 0.1 N NH_3 was used by microorganisms.	24.5 Hence the ∞ of 11.5 cc. 0.1 N NH_3 was used by microorganisms.	22.2 Hence the ∞ of 13.8 cc. of 0.1 N NH_3 was used by microorganisms.
5.9	5.6	5.7	5.4	5.5	5.4
		None.			
		3.4			
		72.6			
		None.			

that the values must obviously be high because of the presence of the other amino-acid or peptone. These amino nitrogen figures are of value because they give some information about the fate of the added amino-acid.

Glycine and Histidine.—The organisms grew distinctly better in a medium containing glycine and histidine than they did in a medium containing only histidine. The histamine production was increased by 6.2 per cent. Most of the glycine nitrogen remained in the histidine fraction in the primary amino condition which indicates that only a small amount of this amino-acid was utilized by the microorganisms.

Arginine and Histidine.—The microorganisms grew very much better in this medium than they did in the one containing glycine and histidine. The histamine production was increased by 10 per cent. The amino nitrogen figures indicate that over half of the arginine was converted into some product that passed into amyl alcohol from a strongly alkaline solution. The arginine may, therefore, also have been decarboxylated.

Peptone and Histidine (*Witte and Difco*).—The presence of either of the above peptones is a great stimulus to the rate of growth of these bacilli, the growth being about equivalent to that obtained in the arginine-histidine medium. The histamine production was increased by 11 per cent in each case.

Alanine and Histidine.—The organisms grew even better in a medium containing alanine than they did in one containing peptone. In this case the histamine production was increased by 20 per cent, the highest value obtained with any of the amino-acids. The alanine used in this work was a mixture of equal parts of the *d*- and *l*-varieties. Two equivalents of this alanine were used. It is interesting to note that one and a quarter equivalents remained, probably as alanine, in the histidine fraction and that a maximum of one-fifth of an equivalent passed into amyl alcohol, possibly as an amine. In short, then, over half of the introduced available nitrogen was consumed by the microorganisms. (We have assumed that *l*-alanine did not serve as a food.)

Leucine and Histidine.—The growth obtained in this case was slightly better than that obtained on the histidine-alanine medium. The histamine production was increased by 18.6 per cent which is almost identical with that obtained on the alanine-histidine

medium. Most of the leucine nitrogen appeared in the histamine fraction which indicates that the leucine was also decarboxylated.

Tryptophane and Histidine.—The growth in this medium was about as dense as that obtained in the peptone-histidine medium; but the histamine production was *decreased*, not increased. The 26.3 per cent of histamine obtained in this case is about 5 per cent less than that obtained in a medium containing histidine as the only amino-acid. We anticipated some trouble with our colorimetric process in this case; but we were relieved to find that tryptophane, or its decomposition products, did not interfere in any way with the color production.

Glutamic Acid and Histidine.—The growth on this medium was slightly less than that on the tryptophane-histidine medium. The presence of glutamic acid *decreases* the histamine production by about 9 per cent. The amino nitrogen figures indicate that most of the glutamic acid nitrogen was still attached to a carboxylated molecule.

Cystine and Histidine.—The growth on this medium was very meager. The presence of cystine decreases the histamine production by 29 per cent so that the formation of this amine is almost nil.

Tyrosine and Histidine.—The fact that tyrosine and its derivatives give a color with alkaline *p*-phenyldiazonium sulfonate, made it necessary for us to modify our usual procedure in this case. The method employed was briefly as follows:

1. The filtered medium, after the usual treatment with 1 cc. of 95 per cent sulfuric acid, was subjected to a distillation, under ordinary pressure, the distillate being collected and examined colorimetrically[10] for volatile phenols. Phenols were absent.

2. The contents of the distilling flask were transferred to a glass dish and evaporated on the water bath. The residue was transferred, with water, to a 25 cc. precision cylinder and diluted to 25 cc. This is the *test liquid.*

3. Of this acid *test liquid* 10 cc. were transferred to a 35 cc. extraction bottle and extracted ten times with ether, using 20 cc. for each extraction. The combined ether extracts were treated

[10] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 235.

with 25 cc. of water, the ether being then removed by distillation at first under ordinary pressures and then *in vacuo*. The solution so obtained was transferred, with water, to a 100 cc. precision cylinder and diluted to 100 cc. It was tested, colorimetrically,[10] for aromatic hydroxy-acids. In this way 3.4 per cent of the tyrosine originally introduced was found to have been converted into oxyphenyllactic acid.

4. The aqueous liquid obtained above, that had been freed from aromatic hydroxy-acids by extraction with ether, was transferred to a 250 cc. Pyrex flask with 90 cc. of water. Silver nitrate, 10 cc. of a 20 per cent solution, was added and the resulting mixture treated with 12 gm. of barium hydroxide in 50 cc. of warm water. The dark brown mixture was filtered, the precipitate being washed with a cold saturated solution of baryta.[11]

This divides the material into two fractions; *the silver precipitate*, which will contain all of the histamine and which should contain most of the histidine, and *the silver filtrate* which should contain all of the tyrosine and tyramine.

5. *The silver precipitate* was suspended in water and treated with an excess of 37 per cent HCl and Na_2SO_4 as has been previously described.[11] The resulting mixture was filtered after 2 hours of digestion on the water bath and the filtrate evaporated on the water bath. The residue was then transferred to an extraction bottle with 10 cc. of water and treated just like any of the histidine test liquids. It was found to contain 11.5 per cent of histidine and 31.4 per cent of histamine. The recovery of imidazoles was only 43 per cent. We have not checked up on the silver precipitation method for histidine sufficiently to be certain that some histidine may not have remained unprecipitated. We are certain that the histamine figure is accurate because the method employed in this experiment was identical with that used in the quantitative precipitation of histamine in some of our earlier work.[11]

6. *The silver filtrate.* The barium and silver ions were exactly removed with H_2SO_4 and HCl, respectively. The filtrate from $BaSO_4$ and AgCl was neutralized with sodium hydroxide and evaporated on the water bath. The residue was transferred to

[11] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 543.

an extraction bottle with 10 cc. of water. The subsequent separation of tyramine from tyrosine and the colorimetric estimation of each substance in its respective fraction was then carried out as described in the following paper.[12] Tyramine was found to be absent. Of the tyrosine originally introduced, 72.6 per cent was recovered.

The histamine production on this medium proceeded at a rate identical with that on a tyrosine-free medium. *Tyrosine neither augmented nor retarded the histamine formation. Tyrosine was not decarboxylated.*

It appears then, that the decarboxylation of histidine is influenced by the presence of other amino-acids in all of the three possible ways. *Tyrosine is without effect. Leucine, alanine, arginine, and glycine increase the rate of decarboxylation. Of these, leucine and alanine are by far the most efficient. Cystine, glutamic acid, and tryptophane decrease the rate of decarboxylation. Of these, cystine is by far the most efficient.* The rate of decarboxylation is not entirely coincident with the rate of growth of the microorganisms because, with the exception of cystine, all of the amino-acids augmented the growth of the organisms; but tryptophane and glutamic acid *decreased* the histamine production.

SUMMARY.

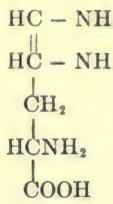
1. The behavior of a large number of microorganisms has been studied on a liquid medium consisting of histidine dichloride (0.2 gm.), ammonium chloride (0.2 gm.), potassium nitrate (0.1 gm.), potassium dihydrogen phosphate (0.4 gm.), sodium chloride (0.8 gm.), sodium sulfate (0.02 gm.), sodium bicarbonate (0.4 gm.), calcium chloride (0.01 gm.), and glycerol (4.0 cc.), in a total aqueous volume of 200 cc.

2. The organisms studied were *Bacillus coli communior* (7 strains), *Bacillus coli communis* (5 strains), *Bacillus lactis aerogenes* (5 strains), *Bacillus acidi lactici* (12 strains), *Bacillus enteritidis*, *Bacillus typhosus*, *Bacillus paratyphosus* A (3 strains), *Bacillus dysenteriæ* Flexner, Morgan, and Shiga, *Bacillus fæcalis alcaligenes* I and III, *Bacillus mucosus capsulatus* (3 strains), *Bacillus bifidus*, *Bacillus influenzæ*, *Bacillus proteus vulgaris* (2 strains), *Bacillus cloacæ*, *Streptococcus hæmolyticus* and *viridans*, pneumococci (Types I, II, III, and IV), and *Bacillus tuberculosis* (5 strains).

[12] Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 193.

3. *Of the twenty-nine strains of coli*—in the narrower sense—that we studied, *six* were able to convert histidine into histamine on our synthetic medium. None of the other organisms were histamine producers.

4. Five members of the colon group—in the narrower sense—gave quantitative evidence that an alkali-stable, carboxylated triamino compound was formed from the histidine. This compound may have the formula



None of the other organisms gave quantitative evidence for the formation of such a compound; but most of them gave results that would lead one to suppose that such a compound was formed to some extent as an intermediate in the decomposition of histidine.

5. Imidazole acetic, propionic, lactic, or acrylic acid was formed by *Bacillus paratyphosus* A (1 strain), *Bacillus dysenteriæ* Flexner, Morgan, and Shiga, *Bacillus fœcalis alcaligenes* I, *Bacillus mucosus capsulatus* (2 strains), and *Bacillus tuberculosis* (4 strains). We cannot say for certain which of the above acids was produced in any case because our method does not differentiate between them.

6. The addition of leucine to the standard medium facilitates the growth of all of the organisms studied.

7. If the organisms produce no histamine when leucine is absent, they do not produce histamine when leucine is present.

8. If the organisms produce histamine when leucine is absent, they produce 20 to 25 per cent more of this amine, in 2 weeks of incubation, when leucine is present. The leucine augments a power that already exists; it does not create a new enzymatic activity.

9. The addition of alanine, leucine, arginine, glycine, or peptone—either Witte or Difco—to the standard medium, augments the growth of the colon bacillus and increases the yield of histamine.

When glutamic acid or tryptophane are added to the standard medium, the growth of the organisms is augmented; but the output of histamine is decreased. Cystine is unfavorable to the growth of the colon bacillus. The presence of this amino-acid reduces the yield of histamine to almost nil. The remarkable retarding influence of cystine on the growth of the microorganisms and on the histamine production by the colon bacillus seems to be due to the hydrogen sulfide which is evolved in quantity during the first few days of incubation. The addition of tyrosine to our standard medium seems to have no influence upon the rate of histamine production.

We wish to acknowledge the technical assistance of Miss Edith H. Bell throughout this work.

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STUDIES ON PROTEINOGENOUS AMINES.

XIII. ON THE ELECTRONIC INTERPRETATION OF CERTAIN BIOCHEMICAL PHENOMENA.

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(Received for publication, October 21, 1921.)

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In our work on biological problems we have frequently encountered reactions that could not have been readily explained on the basis of the accepted structural formulas of the compounds involved, because these formulas are incomplete indices of the degree of oxidation of the carbon atoms. We were led, therefore, to a consideration of the interatomic forces and we have found that the electronic formulas so derived have been of great service in clarifying certain puzzling phenomena.

Parts I and II of this paper contain an electronic explanation of the carboxylase activity of yeast and of the fact that the fatty acids oxidize predominantly in the beta position in the animal body. In Part III we will attempt to show how the electronic formulas for some of the biologically important compounds can be derived.

PART I.

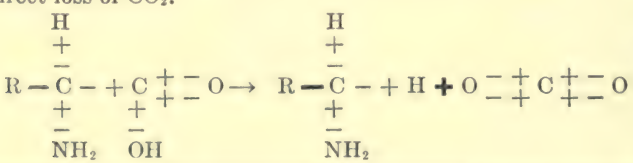
On the Carboxylase Activity of Microorganisms with Particular Reference to Yeasts.

One of the main sources of the formation of amines by the living cell is the decarboxylation of amino-acids. While chiefly accomplished through the activity of microorganisms, there is evidence that this carboxylase activity is also part of the life process of some higher plants and of certain animals. The occurrence of *p*-oxyphenylethylamine in several species of mistletoe[1] (*Phoradendron flavescens*, *Phoradendron villosum*, and *Phoradendron californicum*) and the presence of the same amine in the salivary gland and secretion of some Cephalopoda[2] may be recalled in this connection as evidence for this statement. Up to the present time, however, enzymes capable of decarboxylating amino-acids to amines *in vitro* have not been isolated from either unicellular or multicellular organisms. While the isolation of such an enzyme of the carboxylase type would be of interest, it is doubtful if this accomplishment would clarify the mechanism of decarboxylation any more than the discovery of zymase has enhanced the problem of alcoholic fermentation and of the breakdown of sugars. To obtain a better understanding of the enzymatic activities of the living cell it would seem far more promising to undertake a systematic study of the intermediates formed in the catabolism of higher molecular complexes, especially if we could understand the interatomic forces which govern this breakdown.

The decarboxylation of amino-acids, with the formation of carbon dioxide and the corresponding amine might, theoretically, be accomplished in any of the following ways.

1. With a quadruply positive carboxyl group.

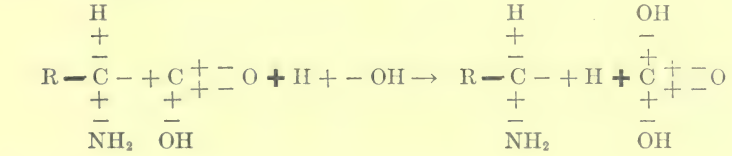
A. Direct loss of CO_2.



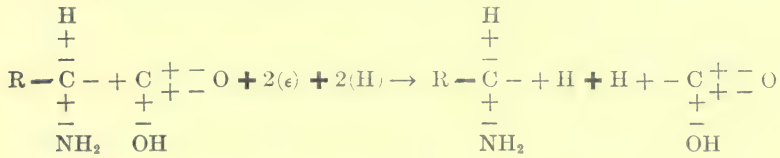
[1] Crawford, A. C., and Watanabe, W. K., *J. Biol. Chem.*, 1916, xxiv, 169.

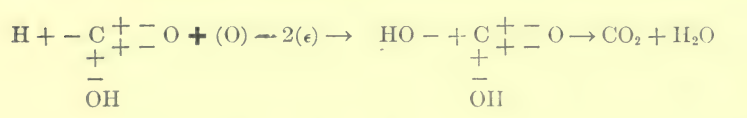
[2] Henze, M., *Z. physiol. Chem.*, 1913, lxxxvii, 51.

B. Hydrolysis.

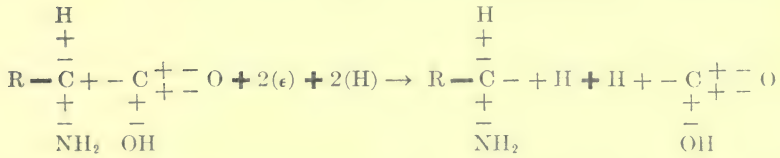


C. Reduction of the quadruply positive carboxyl group.



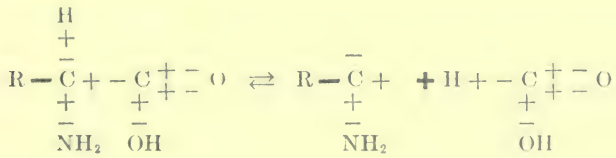


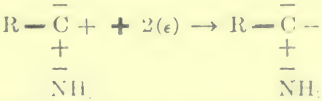
2. With a triply positive carboxyl group.



The formic acid would then give CO_2 and H_2O, by oxidation, as in C above.

The above equation tells nothing about the mechanism of the reaction. Following Nef, we might consider the above reaction to proceed as follows.[3]





[3] Koessler, K. K., *Proc. Inst. Med. Chicago*, 1920, iii, 46.

$$R-\overset{-}{\underset{+}{\underset{-}{C}}}-NH_2 + 2(H+) \rightarrow R-\overset{H}{\overset{+}{\underset{+}{\underset{-}{C}}}}-NH_2 + H$$

For reasons that will be discussed later, the schematic possibility represented by equation (1, C) is hardly worthy of consideration because it involves the reduction of a quadruply positive carbon atom. As far as we now know, only the chlorophyll-containing plants have this power.

A consideration of Types I and II shows that the kind of reaction resorted to must depend upon the direction of the electrical field between the carboxyl group and its neighboring carbon atom. If we were certain that the carboxyl group in all amino-acids was negative with respect to its neighboring carbon atom as in formula (2), we could state with a fair degree of assurance that the decarboxylation of amino-acids must be associated with an oxidation reduction process. Unfortunately, we are at present unable to make a definite statement as to the electrical conditions that prevail in any amino-acid (see under allyl chloride, pages 231 to 233). It seems indeed, as if the charge on the carboxyl group is not the same for all amino-acids.

Although we are, therefore, unable at present to give an electronic interpretation of the carboxylase activity as applied to amino-acids, the electronic point of view offers a fascinating explanation for a related type of carboxylase activity.

Neuberg[4] and his coworkers have shown that yeasts and yeast extracts have the faculty of decarboxylating pyruvic and many other organic acids with the formation of carbon dioxide and aldehydes; *e.g.*, $CH_3-CO-COOH \rightarrow CO_2 + CH_3-CHO$.

Karczag and Breuer[5] have shown that many bacteria, although they ferment pyruvic acid with gas formation, produce no aldehyde. The gas produced, instead of being pure CO_2 as in the case of the yeasts, consists of hydrogen to the extent of 90 per

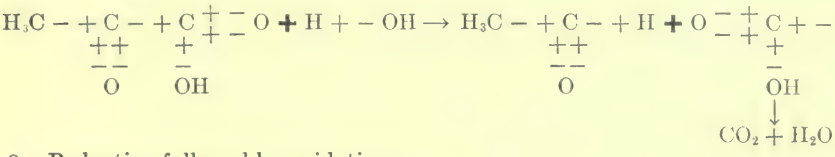
[4] Neuberg, C., and Hildesheimer, A., *Biochem. Z.*, 1911, xxxi, 170. Neuberg, C., and Tir, L., *Biochem. Z.*, 1911, xxxii, 323. Neuberg, C., and Karczag, L., *Biochem. Z.*, 1911, xxxvi, 68, 76; xxxvii, 170. Neuberg, C., and Kerb, J., *Biochem. Z.*, 1912, xlvii, 413, 405.

[5] Karczag, L., and Breuer, E., *Biochem. Z.*, 1915, lxx, 320.

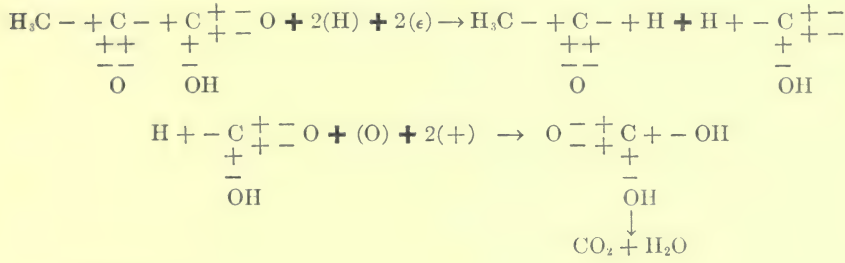
cent. Obviously, then, the action of yeasts on pyruvic acid is radically different from that of the investigated bacteria. The authors believe that some light is shed upon these facts and others to be presented later, by a consideration of the electronic formulas of these substances.

Acetaldehyde and carbon dioxide could be obtained from pyruvic acid by either of the three following processes.[6]

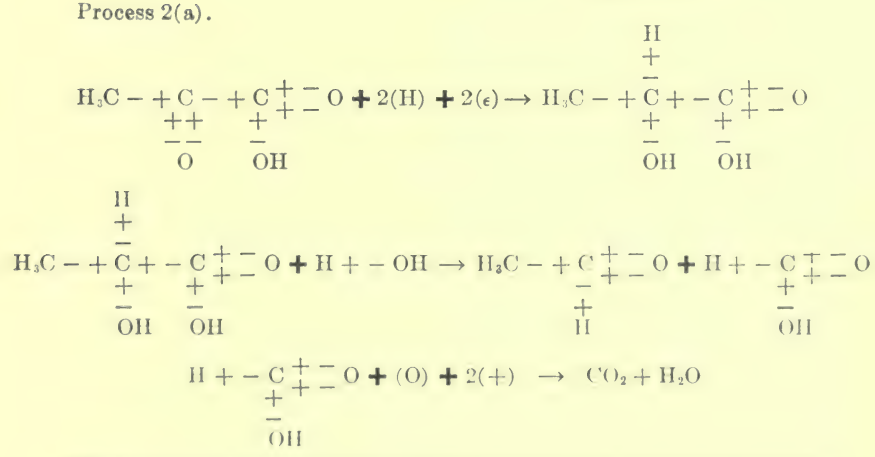
1. Hydrolysis.



2. Reduction followed by oxidation.



Process 2(a).



[6] The proof of the electronic formulas for the compounds used in the equations throughout this paper is given in Part III.

The decomposition of pyruvic acid by yeast *cannot* occur according to process (2) or (2, a) for the following reasons.

Process (2) demands the intermediate formation of formic acid *by the reduction of a quadruply positive carbon atom.* Such a reduction would be equivalent to the direct conversion of CO_2 to formic acid. Up to the present time only the chlorophyll-containing plants have been proved to be capable of reducing CO_2, and they can do so only in the presence of sunlight. This process is, therefore, highly improbable from the start. If we pass lightly over this first serious objection, we come to another that is just as serious. The formulation demands that the formic acid formed at first must then be oxidized to CO_2. That bacteria have this power of oxidation is clearly proved by the work of Omelianski[7] and Pakes and Jollyman.[8] In every case, however, hydrogen is evolved along with the CO_2. Hydrogen is never evolved in the early stages of yeast fermentations. Although the evolution of CO_2 from pyruvic acid by yeast is extremely rapid so that the fermentation is practically over in 24 hours irrespective of the variety of yeast employed, Neuberg and Tir[4] have shown that certain of these yeasts will evolve no gas at all from sodium formate solutions and that none of them evolves more than traces of CO_2 in the course of 24 hours.

An objection might be raised to these conclusions from the work of Neuberg and Tir because these authors used a pure solution of sodium formate in which no real fermentation could occur. It is conceivable that the oxidation of formic acid might take place only in the presence of sugar. Franzen and Steppuhn[9] have shown conclusively that when sodium formate is mixed with a nutrient medium rich in sugar, most of the pure cultures of yeast employed, although they fermented the sugar vigorously, either did not ferment the added formic acid at all or did so only to a very small extent. In no case was the destruction of the formic acid *rapid* enough to conclude that this substance was an intermediate product in the fermentation of sugar. Formulation (2) is, therefore, highly improbable.

[7] Omelianski, W., *Centr. Bakt., 2te Abt.*, 1904, xi, 177, 256, 317.

[8] Pakes, W. C. C., and Jollyman, W. H., *J. Chem. Soc.*, 1901, lxxix, 322, 386, 459.

[9] Franzen, H., and Steppuhn, O., *Z. physiol. Chem.*, 1912, lxxvii, 129.

Process (2, a) demands the intermediate formation of lactic acid. That the reduction of pyruvic acid to lactic acid might be resorted to by *bacteria* in case the pyruvic acid was being used by them as a source of carbon is very possible because as can be seen from the formula, this is an indirect but simple method for converting an *unavailable quadruply positive carboxyl group into one that is triply positive.* That yeasts do *not* resort to this process, in the case of pyruvic acid, is proved by the fact that *lactic acid is not fermented* at all by some yeasts and only to a very slight extent by most (see article by Neuberg and Tir[4] and Buchner and Meisenheimer[10]).

Even if lactic acid were fermented by yeast, the formic acid difficulty would still be insurmountable (see above under process (2)). Apparently then, formula (2, a) cannot represent what happens when pyruvic acid is fermented by yeast.

Process (1) is the only one left and its very simplicity is exactly what one would expect from a reaction that proceeds so smoothly and rapidly. The above considerations have convinced the authors that *the fermentation of pyruvic acid by yeast is accomplished by a purely hydrolytic process.*

From the ordinary structural formula for lactic acid it is difficult to see why a similar hydrolysis does not occur in this case with the formation of CO_2 and alcohol. The hydrolysis of lactic acid with *dilute sulfuric* acid, which takes place quite as readily as the similar hydrolysis of pyruvic acid, gives acetaldehyde and formic acid. The electronic formulas for these two compounds show clearly why CO_2 could not be obtained by the hydrolysis of lactic acid and they *show moreover why yeast cannot hydrolyze lactic acid at all.* Lactic acid contains a *triply* positive carboxyl group while pyruvic acid contains one that is *quadruply* positive. Apparently then, *yeast can hydrolyze only those acids containing quadruply positive carboxyl groups.*

With this idea in mind it is easy to see why oxalacetic acid should give acetaldehyde and CO_2, acetone dicarbonic acid should give acetone and CO_2, and in general why all of the α-ketonic acids should give an aldehyde and CO_2 as primary products because *they all contain quadruply positive carboxyl groups.* A striking confirmation of this statement is found in the fact that *glyoxylic*

[10] Buchner, E., and Meisenheimer, J., *Ber. chem. Ges.*, 1910, xliii, 1773.

acid, the mother substance of the α-ketonic acids, *is practically not fermented by yeast.*[4] *This acid has been shown to contain a negative carboxyl group and it could not give CO_2 on hydrolysis.*

No attempt has been made to explain the mechanism of hydrolysis. Just why pyruvic acid should hydrolyze readily whereas acetic acid must be fused with an alkali to hydrolyze it is not explained by the electronic formulas. The failure of yeast to ferment an acid does not prove that the acid contains a *negative* carboxyl group. On the other hand, if yeast does split CO_2 from an acid *readily* it is fairly safe to conclude that the acid contains a quadruply positive carboxyl group. Acetic acid is fermented very slowly by yeast. Neuberg and Tir make the statement that the gas evolved is only partially absorbed by sodium hydroxide. It would be of interest to ascertain if the unabsorbed gas was methane because if it was methane, the yeast would have proved itself capable of hydrolyzing even this very resistant acid.

PART II.

On the Beta Oxidation of Fatty Acids in the Animal Body.

The work of Knoop, Dakin, and others[11] leaves little doubt that fatty acids undergo oxidation predominantly in the beta position in the animal body. Just why the living cells and hydrogen peroxide should oxidize the beta carbon atom by preference while the halogens react only with the alpha hydrogen atoms of the fatty acids is a question that has been much discussed in the past. It occurred to the authors that this tendency toward beta oxidation in the body (and by hydrogen peroxide) might be closely associated with the electrical structure of the molecule.

The carbon atom can occur in five stages of oxidation; namely,

$$1.\ -\overset{-}{\underset{-}{C}}-\qquad 2.\ -\overset{-}{\underset{-}{C}}+\qquad 3.\ -\overset{-}{\underset{+}{C}}+\qquad 4.\ -\overset{+}{\underset{+}{C}}+\qquad 5.\ +\overset{+}{\underset{+}{C}}+$$

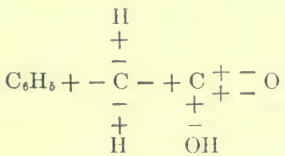
Carbon dioxide is the simplest substance that contains carbon in the completely oxidized condition represented by form (5). Neither microorganisms nor the cells of the animal organism seem to have the faculty of reducing CO_2. Only the chlorophyll-

[11] The references to the original literature are given in Dakin's monograph (Dakin, H. D., Oxidation and reductions in the animal body, London, 1912).

containing plants have been proved to be capable of reducing CO_2 to formaldehyde and they can do so only in the presence of sunlight.[12] Forms (2), (3), and (4) seem to be oxidizable or reducible by living matter depending upon the conditions of the experiment. At present it seems advisable to make no definite statement as to which of these forms is most readily oxidizable.

The work of numerous investigators seems to indicate that form (1) is not *readily* oxidized by living matter. Thus Karczag and Breuer[5] showed that oxalacetic, acetone dicarbonic, and acetic acids were not changed by a large number of microorganisms. Of these acids, acetone dicarbonic and acetic acids contain only quadruply negative and quadruply positive carbon. Oxalacetic acid has one quadruply negative, two quadruply positive, and one triply positive carbon atom. From this it would appear that quadruply negative carbon was not readily oxidized by bacteria.

Although acetic, acetoacetic, and malonic acids, all of which contain only quadruply negative and positive carbon, are completely oxidized in the normal animal body, acetone is attacked with difficulty[13] and phenylacetic acid is not oxidized at all.[14] Acetone has been proved to contain only quadruply positive and quadruply negative carbon and phenylacetic acid must have the formula



It seems therefore, that even the *normal* body cells frequently find it *difficult* to oxidize quadruply negative carbon. From these facts two conclusions can be drawn.

1. If a compound containing quadruply negative carbon together with other more easily oxidizable varieties were fed to a *normal* animal one would expect a predominant and initial oxidation of the more easily oxidizable carbon atoms. In the case

[12] For a critical review of this subject see Bayliss, W. M., Principles of general physiology, London, New York, Bombay, Calcutta, and Madras, 1915, 564.

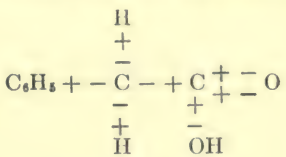
[13] Geelmuyden, H. C., *Z. physiol. Chem.*, 1897, xxiii, 431.

[14] Knoop, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 150.

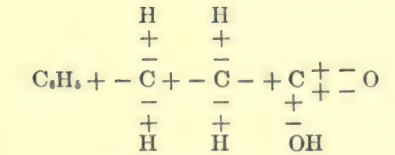
of *most* compounds, these primary oxidation products would then undergo further oxidation, because of the vigorous oxidizing conditions that prevail in the normal organism, so that an excretion of more than traces of these partially oxidized products is hardly to be expected.

2. In certain diseases, notably diabetes, the oxidizing power of the body cells is greatly diminished. Under these conditions the difficultly oxidizable quadruply negative carbon atoms might escape oxidation, at least partially, while the more easily oxidizable carbon atoms in the same molecule might be oxidized. These partially oxidized products, with the quadruply negative carbon still intact, should then be excreted. It is a well known fact that acetone and acetoacetic acid are excreted in large quantities by diabetics. One would expect also that acetic acid, acetone dicarbonic acid, citric acid, and a large number of other substances containing quadruply negative carbon should be at least partially excreted unchanged by diabetics. Citric acid contains one triply positive carbon atom. It might, therefore, be eliminated as acetone dicarbonic acid or if the carboxylase activity was too great it should surely appear as acetone. (The carboxylase of yeast readily decomposes acetone dicarbonic acid into carbon dioxide and acetone by a purely hydrolytic process as has been shown in Part I. A similar decomposition might occur in diabetics.)

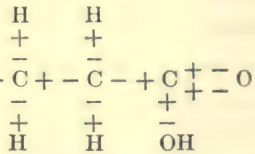
With the idea in mind that quadruply negative carbon is not readily oxidized by the body cells, we can now consider the probable primary oxidation products of a few acids whose electronic formulas shall be repeated here for the sake of simplicity.



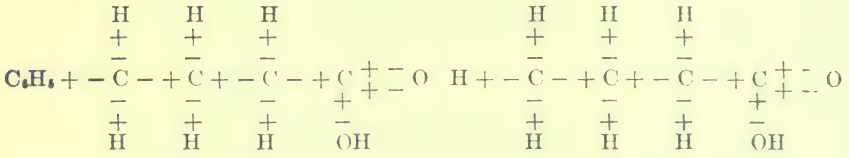
(Phenylacetic acid.)



(Phenylpropionic acid.)



(Propionic acid.)



(Phenylbutyric acid.) (Butyric acid.)

Phenylacetic acid contains the perfectly stable quadruply positive carboxyl group, the very resistant benzene ring, and the presumably difficultly oxidizable quadruply negative alpha carbon atom. This compound should, therefore, not be readily oxidized even by the normal body cells. As a matter of fact phenylacetic acid is *not* oxidized by the normal body cells.

Phenylpropionic acid is *partially oxidized only in the beta position.* This triply negative carbon atom should be the most vulnerable position in the molecule. An oxidation at this point would lead finally to benzoylacetic acid which by one type of hydrolysis would give benzoic acid and acetic acid. Knoop found that phenylpropionic acid was converted into benzoic acid in the normal animal while Dakin was also able to isolate a small amount of benzoylacetic acid under similar conditions.

Phenylbutyric acid *is partially oxidized only in the beta position.* This doubly positive carbon atom should be the most easily attacked position in the molecule. An oxidation at this point would lead to the formation of phenylacetoacetic acid which by one type of hydrolysis would give phenylacetic acid and acetic acid. Knoop found that phenylacetic acid was formed from phenylbutyric acid in the normal animal.

In propionic acid *it is again the beta position that is partially oxidized* and therefore, should be most readily still further oxidized. The final product of the oxidation would be malonic acid. Malonic acid is, however, very readily oxidized by the normal body cells; hence the isolation of a definite end-product is hardly to be expected in the normal animal. As a matter of fact propionic acid is completely oxidized in the normal animal without leaving a clue as to the mode of its decomposition. In diabetics, malonic acid, a polymer of the half aldehyde of malonic acid, or a condensation product of this half aldehyde might possibly be found after feeding propionic acid; but these products seem not to have been sought for.

Butyric acid *is partially oxidized only in the beta position.* Here too, this should be the most vulnerable position. A further oxidation at this point would lead finally to acetoacetic acid which by hydrolysis could lead either to acetone and carbon dioxide or to acetic acid. The increased excretion of acetoacetic acid and acetone after feeding butyric acid to diabetics is a well established fact.

The cited examples would lead one to conclude that the established fact that fatty acids oxidize predominantly in the beta position in the animal body, besides having a chemical verification is the fact that the same type of oxidation occurs with hydrogen peroxide, is exactly what one would expect from the electronic formulas of the fatty acids and their derivatives.

PART III.

Proof of the Electronic Formulas of a Few Biologically Important Organic Compounds.

The General Principles Used in the Determination of Electronic Formulas.

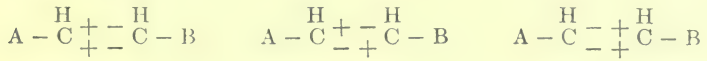
Principle I. The Polarity of a Double or Triple Bond.[15]—Consider an olefine derivative having the following structural formula:

$$\begin{matrix} & H & H & \\ A- & C & = & C-B \end{matrix}$$

---

[15] Falk, K. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1910, xxxii, 1637. Stieglitz has presented this matter in lecture form for the past 6 years, and has applied it to the derivation of the complete formulas for the vinyl derivatives, including acetaldehyde, and for the partial formulas of many organic compounds such as propylene, acrylic acid, crotonic acid, and cinnamic acid. The formula so derived for the vinyl derivatives is $H+-C\overset{-+}{=}C+-X$; for acetaldehyde is $H_3C-+C\overset{+-}{=}O$; for propylene is $H_3C-C\overset{+-}{=}C-+H$; for crotonic acid is $CH_3-C\overset{+-}{=}C-COOH$; and for cinnamic acid is $C_6H_5-C\overset{+-}{=}C-COOH$

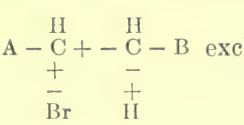
The following three electronic formulas are possible.



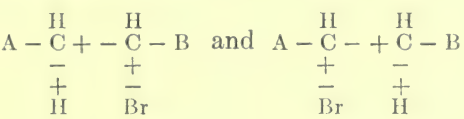
Formula 1. Formula 2. Formula 3.

Which of these is the correct formula can be ascertained by determining how the olefine absorbs compounds of accepted polarity such as H+−OH, H+−Cl, H+−Br, H+−I, H+−NHR, and H+−OR.

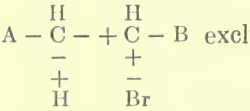
A compound having formula (1) will absorb HBr, for example, to give



A compound having formula (2) will absorb HBr to give a mixture of



A compound having formula (3) will absorb HBr to give



Principle II. The Determination of the Charge on a Carboxyl Group.—Three methods have been employed.

1. In a few cases ketenes are known that have the general formula R−C=C=O. They always have the electronic formula R−C=⁺C⁺=O because they yield acids, anilides, amines, and esters when treated with water, aniline, ammonia, and alcohol, respectively. This is an application of Principle I. In such cases the carboxyl group is quadruply positive.[16]

2. Many carboxylated compounds lose CO_2 when heated. Since CO_2 must have the formula O=⁺C⁺=O, such compounds contain a quadruply positive carboxyl group.[17]

[16] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1730.

[17] Fry, H. S., *J. Am. Chem. Soc.*, 1912, xxxiv, 664; 1914, xxxvi, 248.

3. Some carboxylated compounds split off formic acid when they are heated to 100–150° with dilute sulfuric acid.[18] Since formic acid must have the formula

$$H + - C \begin{matrix} + \\ + \\ - \end{matrix} \begin{matrix} + \\ + \\ - \end{matrix} O$$
$$OH$$

the carboxyl group from which it was derived must be triply positive and it must have been attached to the neighboring carbon atom as follows:

$$C + - C \begin{matrix} + \\ + \\ - \end{matrix} \begin{matrix} + \\ + \\ - \end{matrix} O$$
$$OH$$

4. The above carboxylated compounds give off carbon monoxide when they are warmed gently with fuming sulfuric acid. Carbon monoxide must have the formula $\pm C \pm \mp O$. The carbon is identical, electrically, with the carbon in formic acid. Its evolution has a significance identical with that ascribed to formic acid in the preceding paragraph.

A similar decomposition occurs with phosphoric acid.

The results are less reliable, however, because much higher temperatures are required to start the decomposition.

Principle III. Hydrolysis.—Frequent use has been made of the principle of hydrolysis which has been so thoroughly discussed by others[19] that a repetition seems superfluous.

Assumptions.

1. The charges on the carbon atoms are definitely polarized in at least the large majority of organic compounds.

There has been a tendency in recent years to assume that organic compounds are non-polar.[20] An entirely non-polar com-

[18] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1726.

[19] Nelson, J. M., Beans, H. T., and Falk, K. G., *J. Am. Chem. Soc.*, 1913, xxxv, 1810. Selivanow, *Ber. chem. Ges.*, 1892, xxv, 3517. Stieglitz, J., *Am. Chem. J.*, 1896, xviii, 756. Noyes, W. A., *J. Am. Chem. Soc.*, 1913, xxxv, 769.

[20] For a criticism of this view see Falk, K. G., and Nelson, J. M., *J. Am. Chem. Soc.*, 1914, xxxvi, 209.

pound would be chemically inactive. It may, of course, be true that some or even most of the molecules are really non-polar but such molecules can be of no importance in initiating chemical reactions. *Such inactive non-polar molecules must always be in equilibrium with a certain number of active polar molecules. These active molecules are responsible for the chemical activity of the compound and they are very definitely polar.*[21] The present paper is limited to a discussion of the active polar molecules.

2. The electrical charges hold their places until they are forced to shift because of a definite overwhelming strain. Such shifts are infrequent, belong to classes of compounds rather than to individuals, and must never be assumed to have occurred unless this can be undeniably proved.[22]

References.

It will be necessary to refer frequently to the carbon atoms and to the bonds between the carbon atoms. Instead of placing numbers on the carbon atoms in each of the formulas, which would lead to confusion, the general rule has been adopted of numbering the carbon atoms mentally from *right* to *left* in a horizontal formula and from the top down in a vertical formula. A concrete example will make this clear. Crotonic acid has the structural formula $CH_3-CH=CH-COOH$. The carboxyl carbon is carbon atom 1. and the double bond is the (2–3) bond.

The facts, upon which the electronic formulas are based, can be verified by referring to any large handbook of organic chemistry such as Beilstein's Handbuch der Organischen Verbindungen and Richter's Organic chemistry.

A Consideration of the Formulas.

Propiolic Acid ($H-C\equiv C-COOH$).

When the potassium salt of this acid is heated with water it decomposes smoothly according to the following equation.

[21] Lewis, G. N., *J. Am. Chem. Soc.*, 1913, xxxv, 1448.

[22] Examples of such shifts can be found in the numerous papers by Stieglitz and his collaborators on the electronic interpretation of the Beckmann rearrangement.

$$2\,HC\overset{-}{\underset{-}{=}}C-COOK+H_2O \rightarrow 2\,C_2H_2+CO_2+K_2CO_3$$

Since the carboxyl group is eliminated as CO_2, carbon atom 1 must be quadruply positive.

When propiolic acid is treated with the halogen acids, only the β-halogenated acrylic acids are formed. The exclusive formation of beta derivatives proves that the triple bond is unsymmetrically polar, the electrons being attached to carbon atom 2. The entire formula for propiolic acid, is, therefore,

$$H+-C\overset{+\ -}{\underset{+\ -}{+-}}C-+C\overset{+\ -}{\underset{+}{+-}}O \qquad (1)$$
$$\underset{-}{OH}$$

β-Halogenated Propiolic Acids ($X-C\equiv C-COOH$).

The halogenated propiolic acids decompose readily into $X-C\equiv C-H$ and CO_2. The carboxyl group is, therefore, quadruply positive.

The halogenated propiolic acids react with the halogen acids to give beta derivatives exclusively. Thus β-chloropropiolic acid gives β-dichloroacrylic acid with HCl. This proves that the triple bond is unsymmetrically polar so that the electrons are attached to carbon atom 2. The entire formula for this class of compounds is

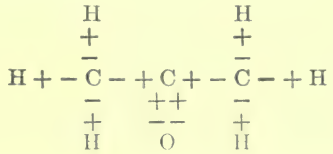
$$X-+C\overset{+\ -}{\underset{+\ -}{+-}}C-+C\overset{+\ -}{\underset{+}{+-}}O \qquad (2)$$
$$\underset{-}{OH}$$

The carbon atoms in these acids are identical, electrically, with the carbon atoms in malonic acid.

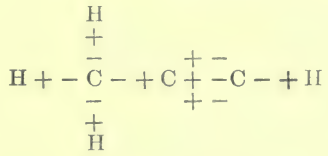
Allylene ($CH_3-C=C-H$).

Allylene is readily absorbed by the halogen acids, the products being $CH_3-CCl_2-CH_3$, $CH_3-CBr_2-CH_3$, and $CH_3-CI_2-CH_3$. In every case the negative halogen attaches itself to the central carbon atom. This unsymmetrical addition of the halogen acids proves that the triple bond is unsymmetrically polar, the electrons being attached to carbon atom 1.

When allylene is heated with dilute sulfuric acid, acetone is formed. Here again the negative oxygen attaches itself to the central carbon atom. Acetone has been proved[23] to have the formula



Both of the end-carbon atoms are quadruply negative. The methyl group in methyl acetylene must, therefore, also be quadruply negative. The entire formula for methyl acetylene is

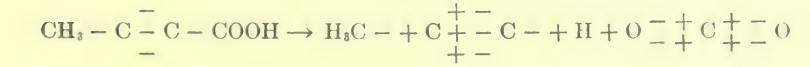


(3)

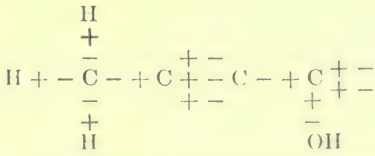
The carbon atoms in allylene are identical, electrically, with the carbon atoms in acetone.

Tetrolic Acid ($CH_3-C\equiv C-COOH$) and Its Relation to Acetoacetic Acid.

When tetrolic acid is heated to 210° it decomposes into allylene and carbon dioxide.



The complete electronic formula for tetrolic acid is, therefore,



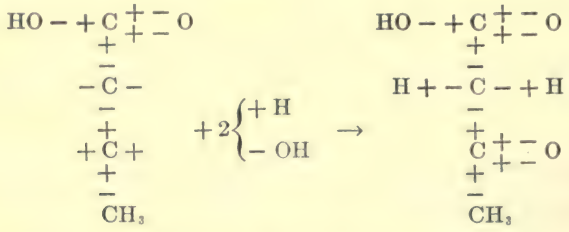
(4)

[23] See foot-note 2. This paper also contains the proof of the formulas for acetic, acetoacetic, acetone dicarbonic, and citric acids.

Since the above decomposition occurs at 210° it alone would not suffice to establish the formula for tetrolic acid. That the above formula is actually correct is proved by the following reactions for tetrolic acid.

It combines with the halogen acids to give β-halogenated tetrolic acids.

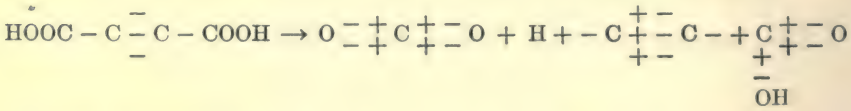
When heated to 105° with a concentrated solution of potassium hydroxide, it decomposes completely with the formation of large quantities of acetone and carbon dioxide and a small amount of acetic acid. This strongly suggests the intermediate formation of acetoacetic acid. This is to be expected from the assigned formula, because the carbon atoms in acetoacetic and in tetrolic acids are electronically identical.



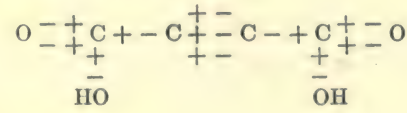
The acetoacetic acid would then give acetone and CO_2 in preponderance, and acetic acid in small amounts as is its habit.

Acetylene Dicarboxylic Acid (*HOOC−C≡C−COOH*).

When this acid is warmed with water it decomposes into propiolic acid and carbon dioxide.



Both carboxyl groups are quadruply positive. The formula for acetylene dicarboxylic acid is



(5)

Acrylic Acid ($CH_2 = CH - COOH$).

The recorded reactions for this substance are:

1. It unites with HCl and HI to give β-chloro- and β-iodopropionic acid, respectively.

2. It gives β-oxypropionic acid, not lactic acid, when heated to 100° with an aqueous solution of sodium hydroxide.

3. When ethyl acrylate is heated with ethyl alcohol containing some sodium ethylate, β-ethoxypropionic acid is formed.

These three entirely one-sided reactions prove that the charges constituting the double bond are polarized so that the electrons are attached to carbon atom 2.

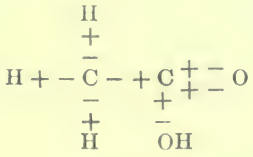
When potassium acrylate is fused with potassium hydroxide, hydrogen, potassium formate, and potassium acetate are formed. This reaction is obviously an hydrolysis at the double bond in which two positive hydrogen atoms are united with the alpha and two negative hydroxyl radicals with the beta carbon atom in exactly the manner that would be expected from the charges assigned to the double bond.

$$CH_2 = CH - COOK + H_2O \rightarrow H_2C = O + CH_3 - COOK$$

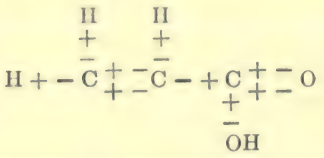
The formaldehyde formed at first reacts in the customary manner with the potassium hydroxide to give hydrogen and potassium formate.

$$H_2C = O + KOH \rightarrow H_2 + HCOOK$$

The most important fact from our point of view, is the formation of acetic acid. The formula for this substance has been proved[23] to be



It contains a quadruply positive carboxyl group. The carboxyl group in acrylic acid must, therefore, also be quadruply positive. This leads to the following complete formula for acrylic acid.

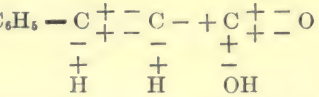


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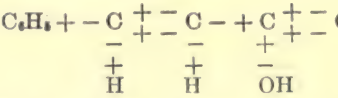
Cinnamic Acid ($C_6H_5-CH=CH-COOH$).

When cinnamic acid is treated with HBr or HI, β-halogenated phenylpropionic acids are formed exclusively. Hypochlorous acid is absorbed so that the negative hydroxyl group attaches itself to the beta carbon atom the product being phenyl α-chloro, β-lactic acid. *The exclusive formation of beta derivatives proves that the double bond* is unsymmetrically polar so that the electrons are attached to the alpha carbon atom.

When cinnamic acid is fused with potassium hydroxide, benzoic acid and acetic acid are the products, the negative OH group being *again*, at this high temperature, attracted *to the beta position.* The formation of acetic acid proves the carboxyl group to be quadruply positive. The formula for cinnamic acid is



The remaining unsolved bond is probably (+−), because cinnamic acid gives ortho and para derivatives with HNO_3 exactly as is the case with phenol, aniline, and the halogenated benzenes in which the substituting group is surely negative. The entire formula for cinnamic acid would then be

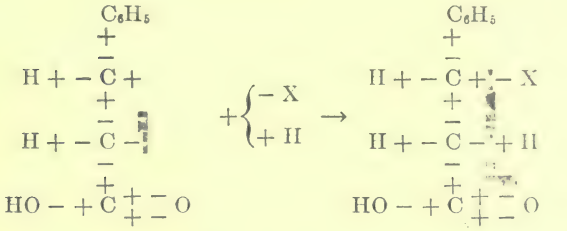


(7)

It is electronically identical with acrylic acid, a positive C_6H_5 group having replaced the positive hydrogen.

β-Chloro-, Brom-, and Oxyphenylpropionic Acid ($C_6H_5-CHX-CH_2-COOH$).

The formulas for these substances follow as a natural corollary from that of cinnamic acid as can be seen from the following equation.



(8) is the formula for β-chloro-, brom-, or oxyphenylpropionic acid.

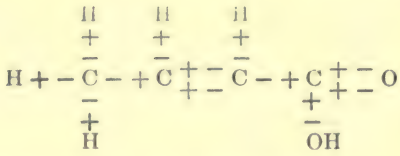
Crotonic Acid ($CH_3-CH=CH-COOH$).

Crotonic acid combines with hydrogen iodide to give β-iodobutyric acid, with hydrogen bromide to give β-brombutyric acid and with sodium ethylate to give β-ethoxybutyric ester. In every case the negative radical attaches itself to the beta carbon atom. The double bond is, therefore, unsymmetrically polar, the electrons being attached to the alpha carbon atom.

When crotonic acid is fused with potassium hydroxide it is hydrolyzed at the double bond to give acetaldehyde and acetic acid. The acetaldehyde formed at first is not liberated as such but reacts with KOH in the customary manner to give acetic acid and hydrogen.

$$CH_3-CH=CH-COOK + KOH \rightarrow CH_3-CHO + CH_3-COOK$$
$$CH_3-CHO + KOH \rightarrow CH_3-COOK + H_2$$

At high temperatures the double bond is still unsymmetrically polar so that the negative OH groups attach themselves to the beta carbon atom. The important fact is the formation of 2 molecules of acetic acid because this proves the polarity of the (1–2) and the (3–4) bond. Acetic acid contains a quadruply positive carboxyl group; so the carboxyl group in crotonic acid must also be quadruply positive. The second molecule of acetic acid, coming as it does from the acetaldehyde formed at first, proves that the (3–4) bond must have an electrical polarity like that of acetaldehyde; namely, (C−+C). The entire formula for crotonic acid is



(9)

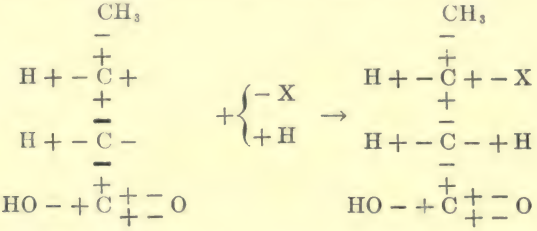
Crotonic acid contains the acrylic acid nucleus.

β-Oxy- and β-Halogenated Butyric Acids ($CH_3-CHX-CH_2-COOH$).

The facts that are needed for the proof of these formulas have already been given under crotonic acid. They will be repeated here for the sake of clearness.

When crotonic acid is heated with an aqueous solution of sodium hydroxide, β-oxybutyric acid is formed.

When crotonic acid is heated with hydrogen bromide or iodide, β-brom- and β-iodobutyric acids are formed, respectively. Electronically these reactions proceed as follows.



(10)

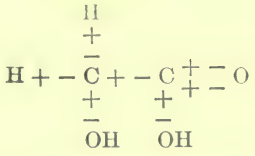
Formula (10) is that of β-oxy, β-ethoxy, and β-halogenated butyric acids. The carbon atoms are electrically identical with the carbon atoms in crotonic acid. It is not surprising, then, to find that they readily lose HX to give crotonic acid.

Glycollic Acid ($CH_2OH-COOH$).

When glycollic acid is warmed with concentrated sulfuric acid it decomposes into carbon monoxide, water, and trioxymethylene.

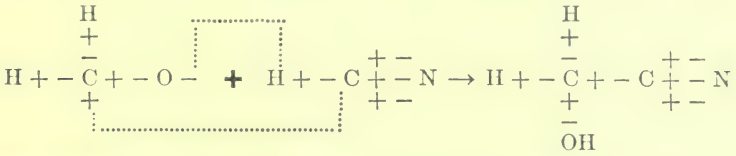
$$3\ CH_2OH-COOH \rightarrow 3CO + 3H_2O + (CH_2O)_3$$

In this case the carboxyl group is split off as carbon monoxide and the formaldehyde formed at first polymerizes to trioxymethylene. The carboxyl group must, therefore, be triply positive. The complete formula for glycollic acid is



(11)

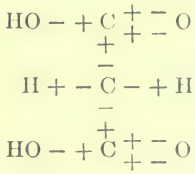
This formula is substantiated by the preparation of glycollic acid from formaldehyde and prussic acid.



The nitrile then gives the acid on hydrolysis which can produce no change in the electrical conditions of the molecule.

Malonic Acid ($COOH-CH_2-COOH$).

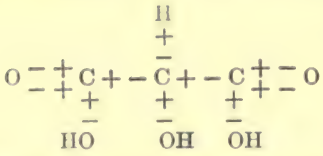
When the acid is heated above its melting point, 132°, it decomposes smoothly into CO_2 and acetic acid. The evolution of CO_2 proves that at least *one* of the carboxyl groups is quadruply positive. Acetic acid has been proved to contain a positive carboxyl group; hence the second carboxyl group in malonic acid must also be quadruply positive. The entire formula for this substance is, therefore,



(12)

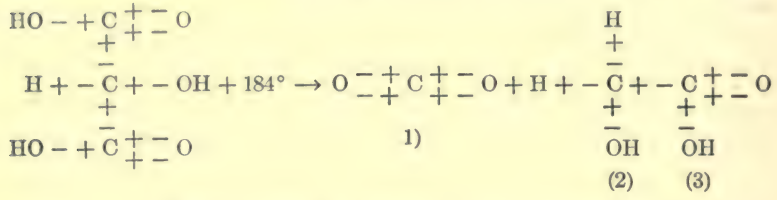
Tartronic Acid ($HOOC-CHOH-COOH$).

When tartronic acid is heated above its melting point, 184°, it decomposes smoothly into carbon dioxide and a polymer of glycollic acid. The evolution of CO_2 proves that *one* of the carboxyl groups is quadruply positive. Glycollic acid has been proved to have a negative carboxyl group. The formula for tartronic acid must then be



(13)

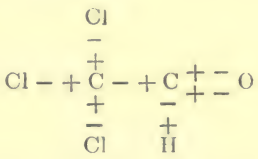
The decomposition can be represented electronically as follows



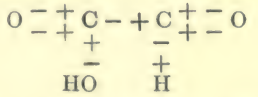
Mesoxalic Acid (*HOOC—CO—COOH*).

When this acid is heated above its melting point it decomposes into carbon dioxide and glyoxylic acid. The evolution of CO_2 proves that one of the carboxyl groups is quadruply positive. The formula for glyoxylic acid cannot be proved from the recorded reactions of that substance; but the following indirect proof seems rather convincing.

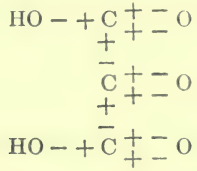
The formula for chloral must be



because it gives chloroform and sodium formate when treated with aqueous alkalies. If it were possible to substitute OH groups for the three negative halogen atoms without separating the carbon atoms, glyoxylic acid would be formed. The formula for glyoxylic acid should then be



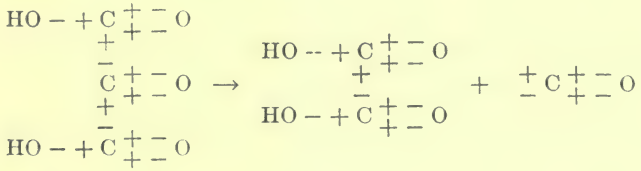
From this one would conclude that one of the carboxyl groups in mesoxalic acid was triply positive and that the entire formula for this substance is



(14)

One assumption has been made; namely, that the substitution of negative oxygen for negative chlorine in chloral has produced no change in the polarity of the charges binding the carbon atoms together.

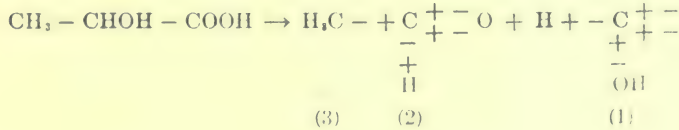
That we were justified in making the above assumption, and that the formula assigned to mesoxalic acid is really correct is proved in a striking manner by the fact that the acid decomposes on boiling with water into carbon monoxide and oxalic acid. Electronically this reaction would be represented as follows.



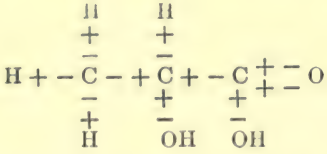
The carbon monoxide might arise from either the central or the triply positive end-carbon atom. In any case *it would be impossible to obtain CO and oxalic acid from a molecule having any but the above electronic structure.*

Lactic Acid ($CH_3-CHOH-COOH$).

When lactic acid is treated at 60° with fuming sulfuric acid, the carboxyl group is eliminated as carbon monoxide. A similar decomposition occurs when lactic acid is heated to 130° with dilute sulfuric acid, the products being acetaldehyde and formic acid.



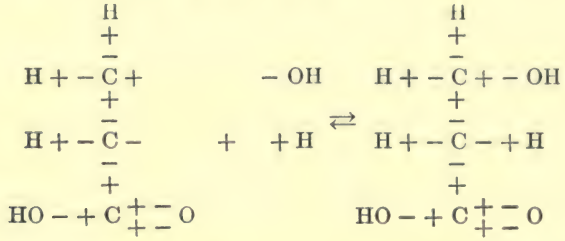
From the above equation the formula for lactic acid is easily seen to be



(15)

β-Oxypropionic Acid (CH_2OH-CH_2-COOH).

When acrylic acid is heated to 100° with an aqueous solution of sodium hydroxide, β-oxypropionic acid is formed. Electronically, this reaction can be written as follows.

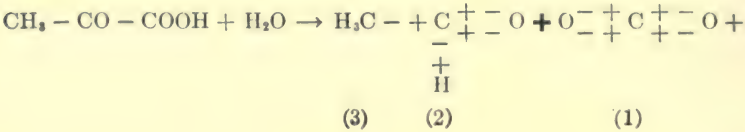


(16)

The carbon atoms in β-oxypropionic acid are identical, electronically, with those in acrylic acid. It is not surprising to find, then that β-oxypropionic acid readily loses water to give acrylic acid. To obtain acrylic acid from *lactic acid*, a far reaching electronic rearrangement is necessary and one would expect that to bring about this transformation, a more drastic treatment would be required. This is the case. The difference in ease of activity between the α- and β-oxy (and halogenated) derivatives is readily explained, in this manner by the electronic formulas.

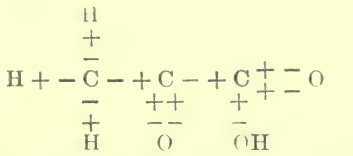
Pyruvic Acid ($CH_3-CO-COOH$).

When pyruvic acid is heated to 150° with dilute sulfuric acid it is hydrolyzed, the products being acetaldehyde and carbon dioxide.



(3) (2) (1)

From the above partially electronic equation the entire formula for pyruvic acid is readily seen to be



(17)

Both pyruvic and oxalic acids contain the electrical dyad $\left(\overset{+}{\ddagger} C - + C \overset{+}{\ddagger}\right)$. They are similarly hydrolyzed by sulfuric acid.

Malic Acid (*HOOC−CH₂−CHOH−COOH*).

When malic acid is heated with dilute sulfuric acid, it decomposes into CO_2, formic acid, and acetaldehyde. When malic acid is warmed with fuming sulfuric acid it decomposes quantitatively according to the following equation.[24]

$$HOOC - CH_2 - CHOH - COOH \rightarrow \underset{\downarrow}{HOOC - CH_2 - CHO} \ (A) + \underset{\downarrow}{HCOOH}$$

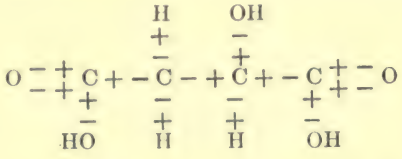
$$C_5H_4O_2 - COOH \quad (B) \quad CO + H_2O$$

Compound (B) is a polymer of (A). When (B) is boiled with dilute sulfuric acid it decomposes into CO_2 and acetaldehyde. Although compound (A) has never been isolated—it polymerizes too readily—its formation as an intermediate product in the decomposition of malic acid by sulfuric acid is proved conclusively by the character of the condensation products formed when malic acid, sulfuric acid, and phenols are heated together.

This reaction proves the entire formula for malic acid. Since CO is evolved—or formic acid with dilute sulfuric acid—*one* of the carboxyl groups must be negative. The negative carboxyl group is attached to carbon atom 1 (see the above equation). The evolution of CO_2 from the polymer of $HOOC-CH_2-CHO$ proves that the other carboxyl group is quadruply positive. The other product is acetaldehyde, a compound of known electronic constitution (CH_3-+CHO).

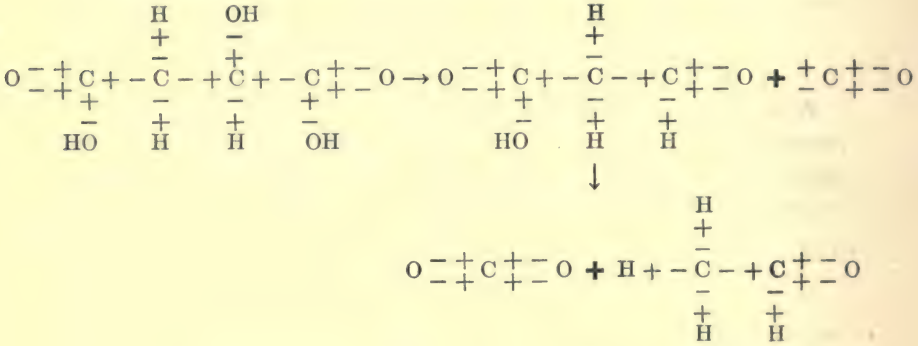
[24] von Pechmann, H., and Welsh, W., *Ber. chem. Ges.*, 1884 xvii, 929, 1649.

The entire formula for malic acid is



(18)

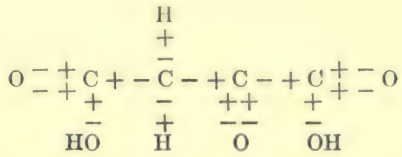
This proof is rendered clear by expressing the decomposition electronically.



Oxalacetic Acid (*HOOC—CH_2—CO—COOH*).

The formula for this substance is clearly proved by the following decompositions of its diethyl ester. When boiled with alkalies it is hydrolyzed so that the products are oxalic acid, acetic acid, and alcohol. When boiled with dilute acids it is hydrolyzed so that carbon dioxide and pyruvic acid are formed. When heated under ordinary pressures it decomposes into carbon monoxide and malonic ethyl ester.

Only one formula can be written that readily explains all of these decompositions; namely,



(19)

As can be seen, an hydrolysis at bond (2–3) would give acetic and oxalic acids; at bond (3–4) would give carbon dioxide and pyruvic acid. The loss of carbon atom 2 would give carbon monoxide and malonic acid.

Tartaric Acid (*HOOC—CHOH—CHOH—COOH*).

Tartaric acid reacts readily with concentrated sulfuric acid or phosphoric acid. The several observers seem to have obtained different products when operating under slightly different conditions. Thus Vangel[25] found that when tartaric acid is heated to 150° with phosphoric acid, equal volumes of CO_2 and CO were formed. He did not examine the non-gaseous residue, nor did he measure the exact quantity of gas obtained per unit weight of tartaric acid. Bouchardat[26] found that when tartaric acid is warmed to 40–50° with fuming sulfuric acid containing 80 per cent of SO_3, a gas is formed that is composed of 4 parts of CO and 1 part of SO_2, and which contains from 2 to 4 per cent of carbon dioxide. He states, moreover, that the carbon dioxide and SO_2 appeared toward the *end* of the reaction. An examination of the non-gaseous residue revealed the presence of some racemic tartaric acid and small amounts of glycollic and pyruvic acids. He gives no data by means of which one could calculate how many of the carbon atoms of tartaric acid were evolved as carbon monoxide. Since these data were necessary to establish the electronic formula for tartaric acid, the following experiment was carried out.

Tartaric acid—1.5000 gm., M.P. 170°—was mixed in a 50 cc. round bottomed flask with 25 cc. of fuming sulfuric acid containing 18 per cent of SO_3. The flask was connected to a 1,000 cc. narrow mouthed precision cylinder that had been inverted and arranged so that the evolved gases could be collected by displacement of water. The flask was heated to 65°. A gas was slowly and steadily evolved that was pure carbon monoxide at first. Not a trace of either SO_2 or CO_2 was present until the gas volume, corrected for temperature, pressure, and aqueous tension was 445 cc. At this point the evolution of gas becomes livelier.

[25] Vangel, B., *Ber. chem. Ges.*, 1880, xiii, 356.

[26] Bouchardat, M. G., *Bull. Soc. chim.*, 1880, xxxiv, series 2, 495.

Considerable SO_2 was present as evidenced by the white fumes that formed when the gas came in contact with the wet walls of the cylinder. The reaction was practically over when about 950 cc. of gas had been collected. The volume shrunk to 840 cc on agitation with water. This removed all of the SO_2 and probably also some of the CO_2. A subsequent agitation with aqueous sodium hydroxide gave a final volume of 800 cc. of gas that proved to be pure carbon monoxide.

The sulfuric acid was colored very pale yellow after the reaction was over so that no charring had occurred.

An examination of this reaction shows the following. Carbon monoxide (445 cc.) was evolved from 1.5 gm. of tartaric acid before either SO_2 or CO_2 were formed. This amount of CO was liberated by the primary action of the sulfuric acid before an oxidation reaction had occurred. This quantity of tartaric acid should give about 225 cc. of CO per carbon atom or 900 cc. if all of the carbon atoms had been converted into CO. The 445 cc. actually obtained represent two carbon atoms, half of the tartaric acid molecule. Since two of the four carbon atoms were evolved as CO *before an oxidation reaction had occurred*, it is fairly safe to conclude that *both* of the *carboxyl groups* were eliminated as CO according to the following equation

$$(CHOH)_2\,(COOH)_2 \rightarrow 2CO + CHO - CHO + 2H_2O$$

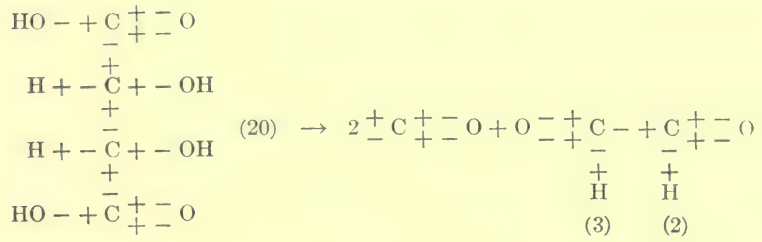
Both of the carboxyl groups must, therefore, have been triply positive. The assumption that glyoxal is an intermediate product in the decomposition of tartaric acid by fuming sulfuric acid is in perfect agreement with the observed fact that all of the carbon atoms in tartaric acid are finally evolved as CO. A primary oxidation of glyoxal would yield glyoxylic acid which would then be converted largely into CO and to a small extent into oxalic acid, CO, and CO_2.

$$1.\begin{cases} CHO - CHO + H_2SO_4 \rightarrow CHO - COOH + SO_2 + H_2O \\ CHO - COOH \rightarrow HCOOH + CO \\ HCOOH \rightarrow CO + H_2O \end{cases}$$

$$2\begin{cases} CHO - COOH + H_2SO_4 \rightarrow COOH - COOH + SO_2 + H_2O \\ COOH - COOH \rightarrow CO + CO_2 \end{cases}$$

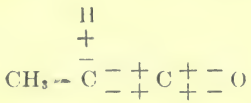
The evolution of so much CO and so little CO_2 could not be explained at all if the assumption were made that even one of the carboxyl groups in tartaric acid was quadruply positive.

The complete electronic formula for tartaric acid and its primary decomposition into CO, glyoxal, and water can be represented as follows.

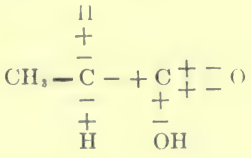


Propionic Acid (CH_3-CH_2-COOH).

That the carboxyl group in propionic acid is quadruply positive is proved by the absorption reactions of methyl ketene, $CH_3-CH=C=O$. Methyl ketene unites with water to give propionic acid, with alcohol to give ethyl propionate, and with ammonia to give propionamide. In every case the negative radical attaches itself to carbon atom 1. This proves that the double bond is unsymmetrically polar; that the partial formula for methyl ketene is

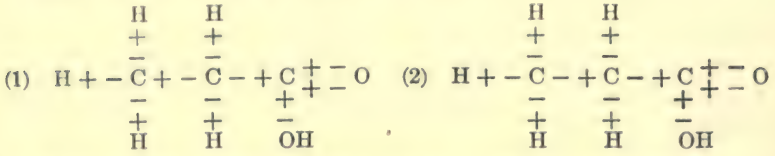


and that propionic acid has the partial formula



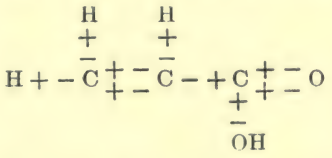
The polarity of the remaining unsolved bond cannot be determined directly. The following partially speculative proofs seem convincing to the authors.

Just two formulas are possible, namely,

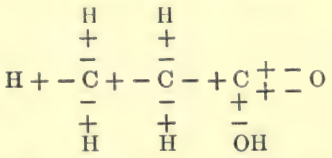


It is, of course, possible that both of these formulas are correct and that the two electrons between carbon atoms 2 and 3 are vibrating so that they are attached first to one and then to the other carbon atom (or they may hold an electron in common). Although the possibility of such an electrometric equilibrium is undeniable, we have no right to assume its existence without proof. Most of the simple compounds examined so far have shown a rigid polarity. In the absence of proof to the contrary we will assume that the same rigidity exists in this compound. There are two proofs that formula (1) is representative of the electrical conditions existing in the molecule of propionic acid.

1. Acrylic acid has been proved to have the formula



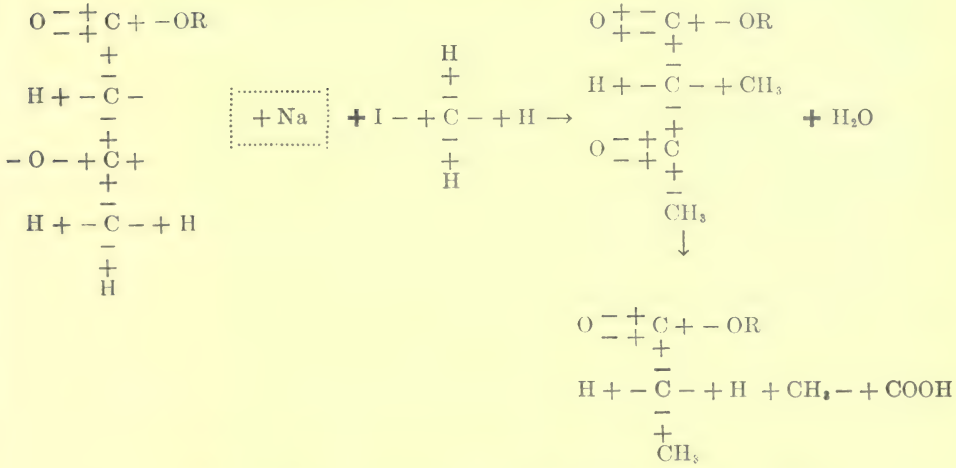
By reduction acrylic acid gives propionic acid. The reduction consists in the addition of two electrons to the beta carbon atom. The formula for propionic acid should then be



(21)

One assumption must be made; namely, that an electronic shift does not take place *within the molecule after the electrons and the hydrogen atoms have been added.*

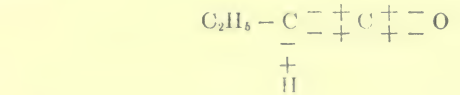
2. Propionic acid can be prepared from the sodium compound of acetoacetic or malonic ester and methyl iodide. The reactions can be represented electronically as follows.



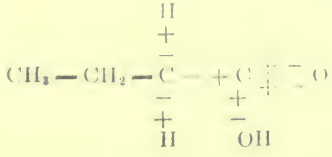
This formula is also obtained by the similar reaction of methyl iodide with the sodium compound of malonic ester and is identical with formula (1).

Butyric Acid ($CH_3-CH_2-CH_2-COOH$).

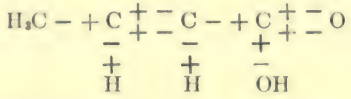
The carboxyl group in butyric acid is quadruply positive. This can be proved from the absorption reactions of ethyl ketene ($CH_3-CH_2-CH\overline{\ }C\overline{\ }O$). Ethyl ketene absorbs water, alcohol, and aniline so that the negative OH, OC_2H_5, and $NH-C_6H_5$ groups attach themselves to carbon atom 1, the products being butyric acid, ethyl butyrate, and butyric acid anilide. These entirely one-sided reactions prove that the double bond in ethyl ketene is unsymmetrically polar so that the electrons are attached to carbon atom 2. The partial formula for ethyl ketene is, therefore,



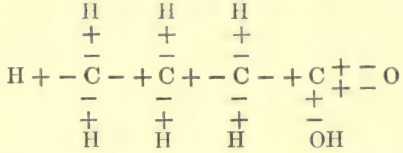
The partial formula for butyric acid must then be



The remaining bonds can be solved only by speculation as follows. Crotonic acid has been proved to have the formula

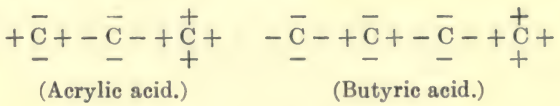


By reduction, crotonic acid gives butyric acid. The reduction consists in the addition of two electrons to the beta carbon atom. The formula for butyric acid should then be

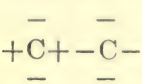


(22)

Although this must certainly be the formula at the moment of reduction, the objection might be raised that the electrical charges could shift after the hydrogen atoms and the electrons had been added. To assume that such a shift did occur would be unreasonable unless the molecule so formed contained electrical dyads of uncertain stability. On the other hand, if the certain stability of every dyad in the molecule could be proved, the above formula should be considered correct until some definite proof to the contrary was found. The undoubted stability of every dyad in this formula is proved by considering the electronic skeleton of the acrylic acid molecule.



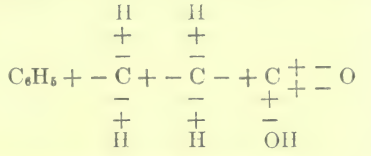
(Acrylic acid.) (Butyric acid.)

The dyad  appears once in the electrically very stable acrylic acid and twice in the assigned formula for butyric acid. The formula assigned to butyric acid is, therefore, not only possible but extremely probable.

Phenylpropionic Acid ($C_6H_5-CH_2-CH_2-COOH$).

The method used in proving the formula for phenylpropionic acid is almost identical with that just described for propionic

acid. A detailed proof is withheld to avoid unnecessary repetition. The formula is



(23)

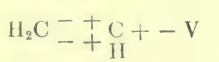
The charge on the benzene ring is given as positive because phenylpropionic acid gives ortho and para derivatives just as is the case with all of the negatively mono-substituted derivatives of benzene. Phenylpropionic acid is electronically identical with propionic acid, a positive hydrogen atom having been replaced by a positive phenyl group.

Two New Electronic Principles.

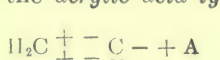
Principle IV.—The polarity of the double bond in an unsaturated aliphatic compound is determined by the electrical charge on the first substituting group.

An examination of the formulas just presented has led us to conclude that the polarity of the double bond in an unsaturated aliphatic compound is determined by the electrical charge on the first substituting group which we will call the directing group.

There are two sharply defined types of unsaturated compounds, those belonging to the *vinyl type*



and those belonging to the *acrylic acid type*



When the first substituent is negative the compound belongs to the vinyl type. Such a compound will always give alpha derivatives by the absorption of a polar compound like HCl. When the first substituent is positive the compound belongs to the acrylic acid type; it gives only beta derivatives by the absorption of polar compounds like HCl.

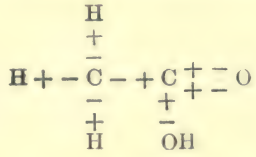
Beside these two simple types, one very stable and common mixed type is possible; namely, V−+C$^{+}_{+}$$^{-}_{-}$C−+A. A com-

pound belonging to this type is both a vinyl and an acrylic acid derivative. Such compounds are electrically very stable because their polarity is fixed by two groups both of which are striving to produce the same electrical configuration.

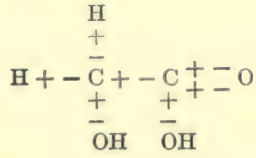
Of the compounds discussed so far, allylene belongs to the vinyl type, propiolic acid to the acrylic acid type, and crotonic, cinnamic, tetrolic, and the β-halogenated propiolic acids belong to the mixed type. The chemical properties of many unsaturated compounds are too incompletely known to make it possible to prove their electronic formulas completely. In such cases Principle IV is frequently of service because it gives us a means of writing a fairly authentic formula on the basis of a few absorption reactions. Examples of such usage will be given presently.

Principle V.—A carbon atom that is attached to an oxygen atom will become at least doubly positive, when this is at all possible.

Glycollic acid is the simplest example of the truth of this principle. The electronic formula for acetic acid is



When one of the paraffin hydrogen atoms is replaced by an OH group, the compound formed, glycollic acid, has the formula



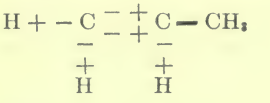
The introduction of one OH group has caused carbon atom 2 to lose *four* electrons. From this it would seem that a triply negative carbon atom is electrically unstable when it is combined with oxygen. The triply negative carbon atom will, when this is possible, lose two electrons to a neighboring carbon atom. Of the compounds discussed so far, citric, tartronic, lactic, malic, and tartaric acids can be cited as illustrations of the truth of this principle. All of these compounds are α-hydroxy-acids and

they all contain a negative carboxyl group. In all of these cases, the alpha carbon atom, which is united with the OH group, is at least doubly positive.

The real value of Principles IV and V lies in the fact that they give us a means of accurately predicting the behavior of certain classes of organic compounds and of establishing the formulas for some compounds whose chemical properties are too incompletely known to make a complete proof of their electronic formulas possible. We will now consider a few such examples.

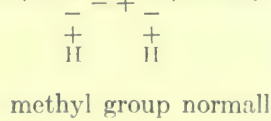
Propylene ($CH_2{=}CH-CH_3$).

In all of its absorption reactions, propylene gives only isopropyl derivatives. Thus HCl, HBr, and HI give isopropyl chloride, bromide, and iodide respectively. Sulfuric acid gives isopropyl sulfate which by hydrolysis gives isopropyl alcohol. This proves that the electrical charges constituting the double bond are unsymmetrically polar, the electrons being attached to carbon atom 3.



The polarity of the remaining unsolved bond cannot be determined directly. That the CH_3 group carries a negative charge is rendered highly probable by the following line of reasoning.

The polarity of the double bond in propylene is like that in the vinyl compounds. Propylene must, then, be a vinyl derivative; hence by Principle IV, the methyl group is negative. The complete formula for propylene is



(24)

That an unopposed methyl group normally carries a negative charge is clearly shown by an examination of some aromatic and aliphatic compounds. Toluene gives ortho and para derivatives almost exclusively just as is the case with phenol and chlorobenzene. The methyl group, like the OH and Cl radicals, functions negatively. Negative methyl groups have also been encountered

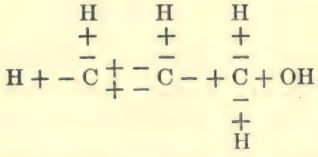
in acetic acid, acetone, acetoacetic acid, tetrolic acid, allylene, acetone dicarbonic acid, and citric acid. Propionic acid is the only compound that we have encountered up to date that *may* contain a positive methyl group.

Allyl Alcohol ($CH_2{=}H{-}CH_2OH$).

When allyl alcohol is heated with a saturated solution of potassium acid sulfite, $CH_2SO_3H-CH_2-CH_2OH$ is formed, the negative SO_3H group going to the beta carbon atom.

When allyl alcohol is treated with hypochlorous acid, $CH_2OH-CH_2Cl-CH_2OH$ is formed, the negative OH group attaching itself to the beta carbon atom.

This proves that the double bond in allyl alcohol is unsymmetrically polar so that the electrons are attached to carbon atom 2. The charge on the CH_2OH group cannot be determined directly; but there is good evidence that it is positive and that the entire formula for allyl alcohol is



(25)

The indirect proof of this statement is as follows.

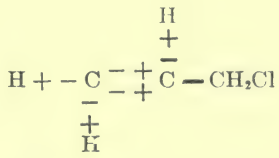
1. Allyl alcohol gives only beta derivatives when it absorbs electrically polar compounds. In this respect it behaves exactly like acrylic acid, and it belongs, therefore, to the acrylic acid type, *not* to the vinyl type. The CH_2OH group should, therefore, be positive like the carboxyl group in acrylic acid (Principle IV).

2. By Principle V, a carbon atom that is attached to oxygen will become at least doubly positive. The oxymethyl group in allyl alcohol should, therefore, be positive.

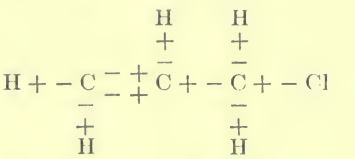
3. To assume that the oxymethyl group was negative would be unreasonable, because if that were true, the polarity of the double bond in allyl alcohol and in propylene should be identical. We can see no reason why an added oxygen atom should change the *remote* electrical charges on the molecule if it were incapable of changing the charge on the carbon atom to which it is attached.

Allyl Chloride ($CH_2{=}H{-}CH_2Cl$).

Allyl chloride combines with hydrogen chloride at 100° to give propylene chloride, $CH_3{-}CHCl{-}CH_2Cl$. Concentrated sulfuric acid is readily absorbed by allyl chloride at room temperatures. When the resulting acid sulfate is treated with water, the product is $CH_3{-}CHOH{-}CH_2Cl$. The negative group attaches itself to carbon atom 2 in each case. These facts prove that for temperature ranges from 20 to 100° the charges constituting the double bond are unsymmetrically polar, *the electrons being attached to the beta carbon atom.* The partial formula for allyl chloride is



The charge on the CH_2Cl group cannot be determined directly; but there is good evidence that it is negative and that the complete formula for allyl chloride is



(26)

The absorption reactions of allyl chloride are identical with those of propylene and the vinyl derivatives but the reverse of those of acrylic acid and allyl alcohol. Allyl chloride is, therefore, a vinyl derivative; the charge on the CH_2Cl group is negative. The CH_2OH group has just been shown to be *positive.* Although the negative oxygen atom will, when possible, repel two electrons from the carbon atom to which it is attached, this seems not to be the case with the negative chlorine atom. Stated differently, when a carbon atom is attached to an oxygen atom the carbon atom will be charged ${}^{+}_{-}C^{+}_{-}$ in preference to ${}^{-}_{-}C^{+}_{-}$, the latter form being rarely capable of existence. *A carbon atom that is attached to chlorine is perfectly stable when it is charged*

$\overline{\underline{\underline{}}}C\underline{\underline{+}}$. This would indicate that triply negative carbon is not *inherently* unstable; it is the attached oxygen atom that renders it so.

The negative charge assigned to the CH_2Cl group is in perfect agreement with its directing force as manifested in the aromatic series. Benzyl chloride gives ortho and para derivatives on halogenation and nitration just as is the case with all of the negatively mono-substituted benzene derivatives.

It might not be out of place at this point to call attention to the need of certain facts that would help greatly to establish the electronic formulas of some biologically very important compounds.

1. Since the CH_2OH group in allyl alcohol is surely positive, it ought also to be positive in benzyl alcohol. Then benzyl alcohol or any of the ethers derived from it should direct the second incoming substituent predominantly to the meta position.

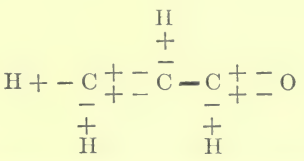
2. It is very important for protein chemistry that we know how the NH_2 group affects the carbon atom to which it is attached. Triply negative carbon has been shown to be stable when attached to chlorine and unstable when attached to oxygen. Although $CH_2OH+-COOH$ is surely the formula for glycollic acid, $CH_2Cl-+COOH$ is probably, therefore, the formula for monochloroacetic acid. What is the formula for aminoacetic acid? This will be hard to prove directly because glycine is an extremely stable compound; but the determination of the following facts would render an indirect proof possible. Does allyl amine give alpha or beta derivatives when it is treated with polar compounds? Does benzyl amine give ortho and para or meta derivatives? From these facts it would be possible to conclude whether the NH_2 group repelled electrons from the carbon atom to which it is attached as is the case with the OH group, or whether triply negative carbon is stable when attached to the NH_2 group as it is when attached to chlorine.

3. Does oxygen repel electrons from the carbon atom to which it is attached *because of its strongly negative character?* This could be determined by examining the properties of certain fluorine derivatives. Since fluorine is more decidedly negative than oxygen, it should be able to repel electrons from the carbon atom to which it is attached at least as certainly as oxygen *if this repulsion was occasioned by the negative character of the fluorine atom.* The oxy-

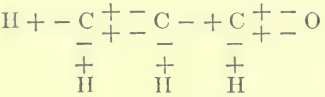
gen atom may prove to be unique in this respect, which might throw some light upon the intra-atomic structure of this atom. The absorption reactions of allyl fluoride and the behavior of benzyl fluoride on nitration, etc., would settle this point.

Acrolein ($CH_2{=}CH{-}CHO$).

When acrolein is treated at low temperatures with hydrogen chloride or hydrogen bromide, β-chloro- and β- brompropionaldehyde are formed respectively. This proves that the electrical charges constituting the double bond are unsymmetrically polar, the electrons being attached to carbon atom 2. The partial formula for acrolein is



The charge on the aldehyde group cannot be determined directly; but there is good evidence that it is positive and that the complete formula for acrolein is



(27)

1. The absorption reactions of acrolein are identical with those of acrylic acid and allyl alcohol; but the reverse of those of the vinyl derivatives. Acrolein, therefore, belongs to the acrylic acid type; the charge on the aldehyde group is positive (Principle IV).

2. Since the charge on the carboxyl group of acrylic acid has been proved to be positive and that on the oxymethyl group of allyl alcohol was proved to be most probably positive, it would be strange indeed if the aldehyde group, which stands between the CH_2OH and the $COOH$ groups in degree of oxidation, should be negative.

3. Aldehyde groups are usually if not invariably positive. In glyoxylic acid, for example, where an aldehyde and a carboxyl group vie for the electron, it is the aldehyde group that is positive. In the aromatic series the aldehyde group directs the second incoming substituent predominantly to the meta position as is the case with nitrobenzene.

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STUDIES ON PROTEINOGENOUS AMINES.

XIV. A MICROCHEMICAL COLORIMETRIC METHOD FOR ESTIMATING TYROSINE, TYRAMINE, AND OTHER PHENOLS.

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(Received for publication, October 21, 1921.)

INTRODUCTION.

The discovery of the diazonium salts by Griess in 1866[1] opened up a most fruitful field for scientific investigation by giving the investigator a class of chemical compounds that are highly susceptible to a large variety of chemical changes. Among these chemical reactions is one that is of particular value because it gives rise to colored compounds; namely, the ability of diazonium compounds to combine (couple) with imidazoles, phenols, and amines in alkaline solutions. *p*-Phenyldiazonium sulfonate, usually called diazobenzenesulfonic acid, is particularly valuable for colorimetric determinations because it is comparatively stable in water solutions, it does not couple with itself to give a highly colored compound, it is easy to prepare, the dyes formed from it are sufficiently soluble in alkaline solutions to render the use of a colorimeter possible, and the colors produced, usually orange to red, are easily compared because they do not readily produce retinal fatigue. It is not surprising then, to find that *p*-phenyldiazonium sulfonate has been used for some time as a qualitative test for imidazoles, phenols, and amines.[2, 3] Up to the present time two methods have been proposed for the use of this diazonium salt in the quantitative estimation of imidazoles;[4, 5]

[1] Griess, P., *Ann. chem. Pharm.*, 1866, cxxxvii, 39.

[2] Ehrlich, P., *Z. klin. Med.*, 1882, v, 285; *Char. Ann.*, 1883, viii, 140; *Deutsch. med. Woch.*, 1883, ix, 549; 1884, x, 419.

[3] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

[4] Weisz, M., and Ssobolew, N., *Biochem. Z.*, 1913, 14, lviii, 119.

[5] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

but as far as is known to the authors this reagent has not been used, heretofore, for the quantitative estimation of phenols. This paper contains a detailed description of methods for the microchemical colorimetric determination of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine.

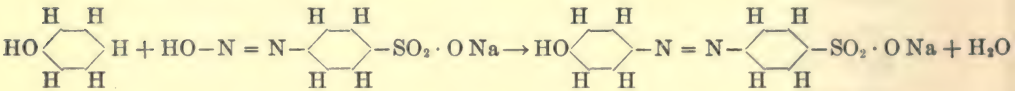
From the standpoint of color development, these phenols can be divided into three classes; namely,

1. Phenols in which the para position is not occupied by a second substituent.

2. Phenols in which the para position is occupied by a second substituent *that does not contain an amino group.*

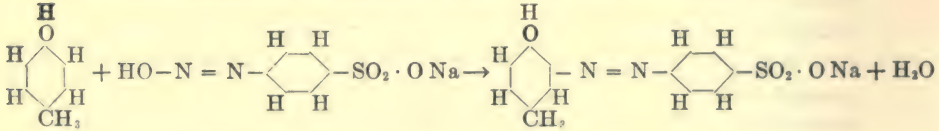
3. Tyrosine and tyramine.

When the position *para* to the phenol radical *is not occupied*, as in phenol, *o*-cresol, and *m*-cresol, the substitution with *p*-phenyldiazonium sulfonate occurs, at least predominantly, in this position.



The coupling is so rapid that about 95 per cent of the color has appeared before a reading can be taken with a Duboscq colorimeter. The color is fully developed in about 2 minutes and it does not change for about 3 to 5 minutes. In these cases the color is predominantly *yellow* with just a dash of red.

When the position para to the phenol radical *is occupied*, as in *p*-cresol and the aromatic hydroxy-acids, the substitution with *p*-phenyldiazonium sulfonate must occur in the ortho position.



The initial rate of coupling in this case, is somewhat slower than that of phenol; but a color of maximum intensity is,nevertheless, obtained in about 3 minutes. The colors produced are predominantly red so that a neutral solution of Congo red can serve as a comparison standard. These colors are stable for from 3 to 5

minutes. The stability of the color and the character of the change that the color undergoes after it has reached its maximum intensity is a fairly accurate index of the composition of the aromatic hydroxy-acid that is present, if only one of these acids is present. This is discussed in detail in the experimental section of the paper.

Tyrosine and tyramine, in small concentrations, show an anomalous behavior toward *p*-phenyldiazonium sulfonate in alkaline solutions. When a solution of tyrosine or tyramine is first added to the alkaline reagent, a pink color begins to develop promptly as in the case of the aromatic hydroxy-acids. After about 30 seconds, however, the color changes sharply to yellow and fades. The intensities of the yellow colors so produced are not directly proportional to the amount of tyrosine or tyramine present. Obviously, then, the usual procedure is worthless for the estimation of either tyrosine or tyramine.

The sharp change in color from pink to yellow suggested a chemical change, perhaps a tautomeric shift. In an effort to stabilize the pink color, or rather to revive it after the initial reaction had gone to completion, we added a small amount of a concentrated solution of sodium hydroxide. The color was intensified and some of the pink tint was restored; but again the color intensities were not directly proportional to the concentration of the phenol. The phenomena noted were so similar to those that one would expect from a compound in which a tautomeric equilibrium existed between a carbonyl form and an enol form that we thought to stabilize the carbonyl derivative, which we believed to be most highly colored, by allowing it to react with hydroxylamine to form an oxime. The addition of hydroxylamine hydrochloride to the alkaline reagent did *not* give rise to an intensification of color; but when sodium hydroxide was added previous to the addition of hydroxylamine, *a very intense bluish red color was produced which was directly proportional to the amount of tyrosine or tyramine added.* Briefly then, the method for estimating either tyrosine or tyramine consists of

1. A reaction between tyrosine or tyramine and *p*-phenyldiazonium sulfonate in an alkaline—sodium carbonate—solution which gives rise to a primary yellow color. This reaction is allowed to proceed for 5 minutes.

2. The addition of 2 cc. of 3.0 N NaOH, after the initial reaction period of 5 minutes, which intensifies the color, stops any further action between the phenol and the diazonium salt and converts the diazonium salt into a sodium diazotate which cannot couple with the hydroxylamine that is to be added later. The alkali is allowed to act for 1 minute.

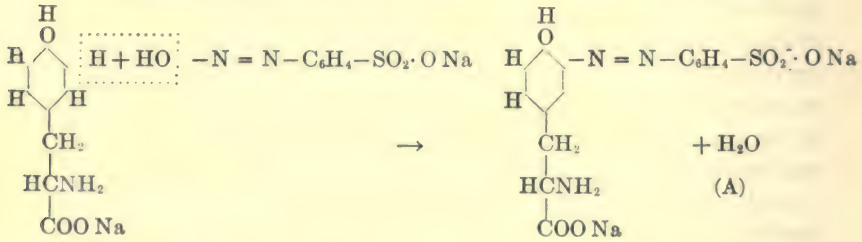
3. The addition of 0.10 cc. of a 20 per cent solution of hydroxylamine hydrochloride, which gives rise to a very intense bluish red color that is stable for at least half an hour.

An explanation of the chemical reactions involved must account for the following facts: Tyrosine and tyramine are the only phenols, that we have examined, that give a color intensification with sodium hydroxide and hydroxylamine. Since oxyphenyllactic acid, which is structurally identical with tyrosine excepting that an OH group replaces the NH_2 group, does not give a color intensification with NaOH and NH_2OH, *the NH_2 group must play a rôle in the color development with these phenols.*

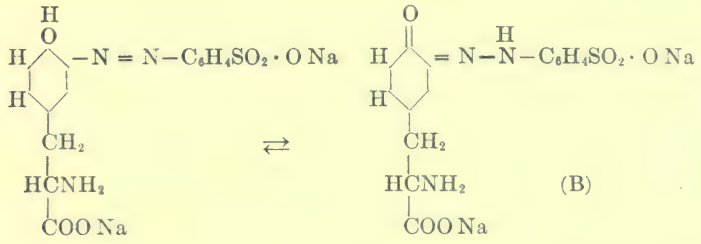
A number of other compounds in which a tautomeric equilibrium exists between a carbonyl form and an enol form, for example acetaldehyde, acetone, and acetoacetic acid, also give an intense color under the conditions just described. The color produced is almost identical with that obtained with tyrosine or tyramine.

We offer the following explanation tentatively for these color phenomena. Work is now under way to determine the correctness of the following formulations.

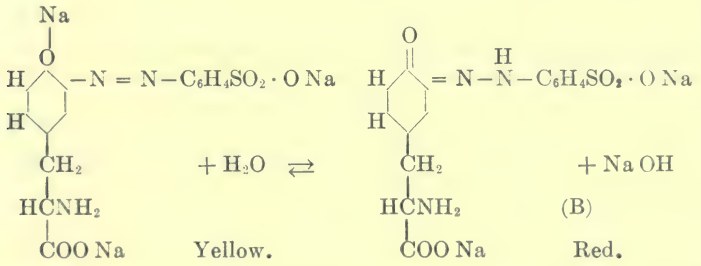
Since the position para to the phenol group in tyrosine and tyramine is occupied by an alanyl side chain, the substitution with diazobenzenesulfonic acid must occur in the ortho position.



Compound A—the yellow phenol form—would be in tautomeric equilibrium with a small amount of the supposedly red quinoid form



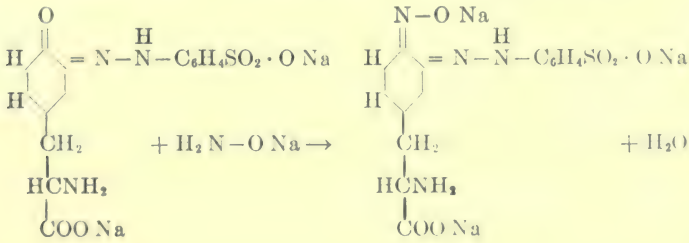
In a solution made alkaline with sodium carbonate, practically all of the compound would exist in its phenol form A. The addition of a strong alkali, like sodium hydroxide, would give rise to the sodium phenate derivative of A which, because of its high degree of dissociation, would pass more easily into the quinoid derivative than the phenol itself.



The result would be an intensification of color with the influx of red. The amount of quinoid derivative formed under the influence of alkali must, nevertheless, be *small* because the color intensification is not very great. The addition of hydroxylamine hydrochloride to such a strongly alkaline solution would give rise to the following reactions.

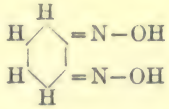
1. $NH_2OH \cdot HCl + 2\,NaOH \rightarrow H_2N-O\,Na + NaCl + H_2O$

2.



One would expect the quinoneoximehydrazone derivative (C) formed in this way to be dark red. Such *o*-quinoneoximehydrazones seem not to have been studied; but the para derivatives have been known for some time.[6] *They all give deep red alkaline solutions.*

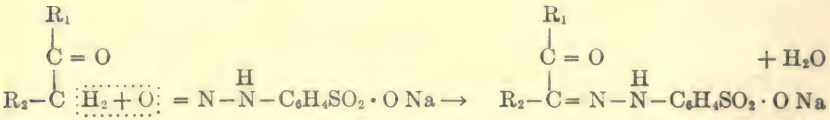
o-Quinonedioxime



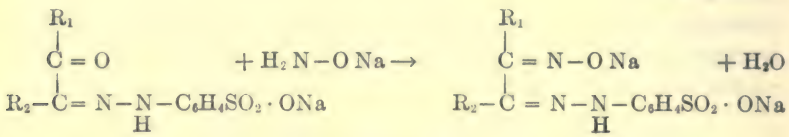
which is a closely related compound, also gives a deep red alkaline solution.[7]

Just why tyrosine should give a highly colored quinoneoximehydrazone while *p*-oxyphenyllactic acid does not give such a highly colored derivative, is a problem for future investigation. The rôle played by the amino group in the side chain of tyrosine and tyramine is still obscure.

The reactions that probably occur when certain aldehydes and ketones are treated with diazobenzenesulfonic acid, sodium hydroxide, and hydroxylamine can be represented by the following type equations.



Pale yellow.

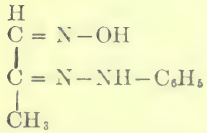


We believe that the red compounds formed with acetaldehyde, acetone, and acetoacetic acid are the sodium salts of hydrazoxime derivatives. Von Pechmann and Wehsarg[8] have prepared a hydrazoxime derivative of methyl glyoxal which is similar to the compound that we believe to be formed with acetone.

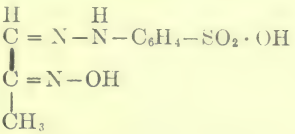
[6] Borsche, W., *Ann. chem. Pharm.*, 1907, cccIvii, 171.

[7] Hantzsch, A., and Glover, W. H., *Ber. chem. Ges.*, 1907, xl, 4344.

[8] von Pechmann, H., and Wehsarg, K., *Ber. chem. Ges.*, 1888, xxi, 2996.



von Pechmann and Wehsarg's hydrazoxime.



Hydrazoxime formed from acetone.

They give no data on the color of the compound in alkaline solutions although they found the alcoholic solution to be yellow and the sulfuric acid solution to be dark red.

EXPERIMENTAL.

Reagents Employed.

Most of the reagents employed in these estimations are identical with those employed by us in the estimation of imidazoles.[5] A detailed description is repeated here for the sake of completeness.

Stock Sulfanilic Acid.—Sulfanilic acid (4.5 gm.) is mixed with 45 cc. of 37 per cent hydrochloric acid (sp. gr. 1.19) in a 500 cc. volumetric flask. Water is then added to the mark. The solid dissolves slowly; but completely.

Stock Sodium Nitrite.—25 gm. of 90 per cent sodium nitrite are dissolved in water and diluted to 500 cc. in a volumetric flask.

Sodium Carbonate.—Baker and Adamson's anhydrous sodium carbonate (5.50 gm.) is dissolved in water and diluted to exactly 500 cc. We recommend the above grade because we have found it to give uniform results. The purity of this carbonate is a very important factor in the color development. Some grades of carbonate contain impurities that give yellow colors of inferior intensities. The finished carbonate solution must be preserved in a glass vessel that has little tendency to dissolve in alkali. Pyrex glass vessels have proved to be entirely satisfactory.

Stock Methyl Orange.—Vacuum-dried Grübler's methyl orange (0.5000 gm.) is dissolved in water and diluted to exactly 500 cc. This solution keeps indefinitely.

Stock Congo Red.—Vacuum-dried Grübler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volumet-

ric flask. Water is then added to the mark. This solution keeps indefinitely.

Stock Acid Fuchsin.—Vacuum-dried Harmer's acid fuchsin (2.5000 gm.) is dissolved in water and diluted to exactly 500 cc. in a volumetric flask. The thymol-preserved solution keeps indefinitely.

Stock Phenol Red.—Hynson, Westcott and Dunning's phenol red (0.0500 gm.) is dissolved in water and diluted to exactly 500 cc. The thymol-preserved aqueous solution can be kept for at least 1 year. The alcoholic solution employed by many investigators for pH determinations deteriorates quite rapidly and cannot be used in this work.

Standard Indicator Solutions.—For the estimation of phenol, a solution containing 10 cc. of stock phenol red in a total aqueous volume of 100 cc. is employed. Redistilled water should be used for dilutions and the volumetric flask should be thoroughly rinsed with distilled water before the phenol red solution is introduced. It is best to prepare a fresh standard every day. The color so obtained matches that produced by phenol perfectly. When this standard indicator solution has been used for comparisons the symbol (Ph−R) is suffixed to the reading obtained.

For the estimation of o- and m-cresol, a solution containing 5 cc. of stock methyl orange in a total aqueous volume of 500 cc. is employed. The color so obtained matches that produced by *o*- and *m*-cresol almost perfectly. When this standard indicator solution has been used for comparisons the symbol (MO) is suffixed to the reading obtained.

For the estimation of p-cresol, and p-oxyphenylacetic, p-oxyphenylpropionic, and p-oxyphenyllactic acids, a solution containing 1.00 cc. of stock Congo red in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (CR) is suffixed to the reading obtained.

For the estimation of tyrosine or tyramine, a solution containing 1 cc. of stock acid fuchsin and 1.8 cc. of stock methyl orange in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (F−MO) is suffixed to the reading obtained.

Preparation of p-Diazobenzenesulfonic Acid Solution (The Reagent).

1.50 cc. each of the stock sulfanilic acid and sodium nitrite solutions are measured into a 50 cc. volumetric flask. The flask is then immersed in an ice bath for 5 minutes. Then 6.00 cc. more of the stock sodium nitrite solution are added and the well mixed solution is again allowed to lie in the ice bath for 5 minutes. Distilled water is then added to the mark and the flask returned to the ice bath where it is kept. This reagent must not be used for at least 15 minutes after diluting with water. We have found it to give perfect results after 24 hours. It is best, however, to prepare a fresh reagent every day.

Preparation of the Phenols.

Phenol.—Merck's highest purity phenol was distilled *in vacuo*. The product collected boiled at 95° in a vacuum of 10 mm. of Hg. The colorless oil solidified readily. The colorless crystals so obtained had a melting point of 43°. The substance was assumed to be 100 per cent pure phenol.

o-Cresol.—A chemically pure product obtained from a local supply house was doubly distilled *in vacuo*. The colorless oil obtained boiled at 81° at a pressure of 10 mm. and at 188° under atmospheric pressure. The oil solidified on standing. The colorless crystals so obtained had a melting point of 32°. It was assumed to be 100 per cent pure *o*-cresol.

m-Cresol.—A chemically pure product obtained from a local supply house was doubly distilled *in vacuo*. The colorless oil so obtained boiled at 104° under 10 mm. pressure and at 201° under atmospheric pressure. It did not solidify. It was assumed to be 100 per cent pure *m*-cresol.

p-Cresol.—The *p*-cresol was obtained as a by-product in the preparation of tyramine.[9] The substance boiled at 90–91° at a pressure of 10 mm. and at 198°under atmospheric pressure. The oil solidified after standing for some time. The colorless crystals so obtained melted at 36°. It was assumed to be 100 per cent pure *p*-cresol.

[9] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 585.

Oxyphenylacetic Acid.—This substance was obtained as a by-product in the preparation of tyramine.[9] The colorless solid had the following properties.

1. It melted at 150°.
2. It left no residue on ignition.
3. The solid (0.1000 gm.) was dissolved in 10 cc. of water and titrated with 0.1 N NaOH using phenolphthalein as indicator. Exactly 6.59 cc. of the alkali were required to produce the first change in the indicator. The amount demanded by the formula is 6.58 cc. The substance was, therefore, 100 per cent pure.

Oxyphenylpropionic Acid.—This compound was prepared synthetically as follows.

Cinnamic acid was reduced, with sodium amalgam, to hydrocinnamic acid.[10]

p-Nitrophenylpropionic acid was then prepared by a modification of the method of Beilstein and Kuhlberg.[11]

Stöhr[12] prepared *p*-aminophenylpropionic acid by reducing the ethyl ester of *p*-nitrophenylpropionic acid with zinc and hydrochloric acid. He isolated the zinc double salt of the acid and treated this with acid and sodium nitrite to prepare *p*-oxyphenylpropionic acid. We reduced *p*-nitrophenylpropionic acid (6 gm.) directly with zinc (14 gm.) and hydrochloric acid (50 cc. of the 37 per cent acid) in alcoholic solution (70 cc. of 95 per cent alcohol) adding the acid slowly and keeping the temperature below 30°. The colorless liquid obtained, after the reaction had been allowed to proceed for 24 hours, was treated with 23.5 cc. of 95 per cent sulfuric acid and subjected to a distillation *in vacuo* at 60°. The pale yellow solid so obtained was dissolved in water (200 cc.) treated with sulfuric acid (20 cc. of the 95 per cent acid) and cooled in an ice bath. Sodium nitrite (60 cc. of a 5 per cent solution) was poured into the cold liquid. The resulting solution was allowed to remain in the ice bath for 1 hour after which it was treated with 250 cc. of water and heated to the boiling point. There was a copious evolution of nitrogen; but practically no tar formation. The aqueous solution was

[10] Fischer, E., Anleitung zur Darstellung organischer Präparate, Brunswick, 8th edition, 1908, 39.

[11] Beilstein, F., and Kuhlberg, A., *Ann. chem. Pharm.*, 1872, clxiii, 132.

[12] Stöhr, C., *Ann. chem. Pharm.*, 1884, ccxxv, 60.

cooled and extracted with ether. Removal of the ether, by distillation, left a pale yellow crystal cake weighing 4.5 gm. which was recrystallized first from water and then from hot benzene. The colorless solid finally obtained had the following properties.

1. It melted sharply at 130° (corrected).
2. It left no residue on ignition.
3. 0.166 gm. of the vacuum-dried solid was dissolved in water and the solution titrated with 0.20 N NaOH using phenolphthalein as indicator. Exactly 5.00 cc. of alkali were required. This is the amount demanded by the formula. The solid was 100 per cent pure.

p-Oxyphenyllactic Acid.—This was prepared from tyrosine by the method of Kotake.[13] The colorless solid obtained after drying to constant weight at 105° had the following properties.

1. It melted sharply at 172° (corrected).
2. It left no residue on ignition.
3. 0.25 gm. was dissolved in water and the solution titrated with 0.1 N NaOH using phenolphthalein as indicator. Exactly 13.70 cc. of alkali were required, which is almost exactly the amount (13.74 cc.) required by the formula. The solid was 100 per cent pure.

Tyrosine.—The tyrosine used in this work was prepared from horn. The pure white, asbestos-like solid had the following properties.

1. It did not melt at a temperature of 290°C.
2. It was free from ammonia.
3. It left no residue on ignition.
4. It contained no cystine.
5. The ammonia obtained from 0.1000 gm. of the solid—Kjeldahl method—neutralized 5.50 cc. of 0.10 N HCl as compared with 5.52 cc. required by the formula. The substance was 100 per cent pure.

Tyramine Hydrochloride.—The synthetic preparation and properties of this compound have been previously described by us.[9] The product used was 98.14 per cent pure tyramine hydrochloride. The impurity consisted entirely of sodium chloride.

[13] Kotake, Y., *Z. physiol. Chem.*, 1910, lxv, 398.

Procedure for Estimating Phenols Other than Tyrosine or Tyramine.

Process I.

The method used in developing the tables as well as the general procedure for estimating phenols other than tyrosine and tyramine is illustrated by the following example.

(1–X) cc. of water and 5 cc. of the 1.1 per cent sodium carbonate solution are accurately measured into the right-hand cylinder of a Duboscq colorimeter. 2 cc. of reagent are measured into a 5 second delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder are then thoroughly mixed by allowing the liquid to flow repeatedly up the inclined tube as far as safety from loss will permit. The mixing should not take over 30 seconds. X cc. of the phenol solution is allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder are mixed *thoroughly* as above.[14] The test cylinder is then transferred to the colorimeter and set at 20 mm. The left-hand cylinder, which should contain the appropriate standard indicator solution is then adjusted constantly until a maximum reading has been obtained.

The most accurate readings can be obtained by choosing such an amount of phenol solution that the standard indicator-containing cylinder has to be set at from 5 to 20 mm.

The method described here can be used on quantities of the phenol-containing solution varying from 0.01 to 1 cc. The combined volume of water and phenol solution used should always be 1 cc. Thus, if 0.10 cc. of the phenol solution is to be used, 0.90 cc. of water is added to the test cylinder. Then X equals 0.10 cc. and 1–X equals 0.90 cc.

Estimation of Small Amounts of Phenol.

A stock 1.00 per cent phenol solution was prepared by dissolving 2.0000 gm. of the pure solid in water and diluting to exactly 200 cc. From this the standard solution was prepared by dilut-

[14] Process II described on page 257 is the same as Process I up to this point.

ing 1 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of phenol.

The color produced with phenol is very intense. It develops so rapidly that it has reached 95 per cent of its maximum value before a reading can be taken. A maximum color is obtained within 2 minutes of the time that the phenol is added to the alkaline reagent. The color remains of maximum intensity for about 5 minutes, then fades slowly without changing appreciably in tint. Most people find this greenish yellow color hard to compare, without practice, because of the speed with which it produces retinal fatigue. Accurate readings can only be obtained by observing the colors briefly, setting the cylinder rapidly and making the fine adjustment after a brief interval of rest. When this is done the readings can be checked with an accuracy of about 1 per cent.

The previously described (Ph–R) standard indicator solution was used for comparisons in the compilation of Table I. This table shows clearly that the color production is directly proportional to the amount of phenol used.

Where only a few determinations are to be carried out it is usually simpler to compare the color produced by an unknown with that produced by a measured amount of a standard phenol solution, the two colors being prepared simultaneously. When the amount of standard phenol used is nearly equal to that in the test liquid, very accurate results can be obtained by this method.

Estimation of Small Amounts of o-Cresol.

A stock 0.1 per cent *o*-cresol solution was prepared by dissolving 1.0000 gm. of the pure solid in water and diluting to exactly 1,000 cc. From this the standard solution was prepared by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *o*-cresol.

The color produced with *o*-cresol is very intense. Its speed of development is like that of phenol. The color, which is predominantly yellow but which contains slightly more pink than that produced by phenol, remains of maximum intensity and permanent tint for about 3 minutes; then it fades rapidly and acquires a redder tint. This color is easier to compare than that of phenol.

TABLE I.

Estimation of Small Amounts of Phenol.

Depth of indicator solution (Ph—R) required to match the color in the test cylinder.	Phenol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.3	0.000002
3.5	0.000003
4.6	0.000004
5.8	0.000005
6.9	0.000006
8.1	0.000007
9.2	0.000008
10.4	0.000009
11.5	0.000010
12.7	0.000011
13.8	0.000012
15.0	0.000013
16.1	0.000014
17.3	0.000015
18.4	0.000016
19.6	0.000017
20.7	0.000018
21.9	0.000019
23.0	0.000020
24.2	0.000021
25.3	0.000022
26.5	0.000023
27.6	0.000024
28.8	0.000025
29.9	0.000026
31.1	0.000027
32.2	0.000028
33.4	0.000029
34.5	0.000030

The previously described (MO) standard indicator solution was used for comparisons in the compilation of Table II. This table shows clearly that the color produced is directly proportional to the amount of *o*-cresol used.

TABLE II.

Estimation of Small Amounts of o-Cresol.

Depth of indicator solution (MO) required to match the color in the test cylinder.	o-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.4	0.000002
3.6	0.000003
4.8	0.000004
6.0	0.000005
7.3	0.000006
8.5	0.000007
9.7	0.000008
10.9	0.000009
12.1	0.000010
13.3	0.000011
14.5	0.000012
15.7	0.000013
16.9	0.000014
18.1	0.000015
19.4	0.000016
20.6	0.000017
21.8	0.000018
23.0	0.000019
24.2	0.000020
25.4	0.000021
26.6	0.000022
27.8	0.000023
29.0	0.000024
30.2	0.000025
31.5	0.000026
32.7	0.000027
33.9	0.000028
35.1	0.000029
36.3	0.000030

Estimation of Small Amounts of m-Cresol.

A stock 0.10 per cent solution of *m*-cresol was prepared by dissolving 1.000 gm. of the colorless oil in water and diluting to exactly 1,000 cc. From this the standard solution was prepared

by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *m*-cresol.

TABLE III.

Estimation of Small Amounts of m-Cresol.

Depth of indicator solution (MO) required to match the color in the test cylinder.	*m*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
1.0	0.000001
1.9	0.000002
2.9	0.000003
3.9	0.000004
4.9	0.000005
5.8	0.000006
6.8	0.000007
7.8	0.000008
8.7	0.000009
9.7	0.000010
10.7	0.000011
11.6	0.000012
12.6	0.000013
13.6	0.000014
14.6	0.000015
15.5	0.000016
16.5	0.000017
17.5	0.000018
18.4	0.000019
19.4	0.000020
20.4	0.000021
21.3	0.000022
22.3	0.000023
23.3	0.000024
24.3	0.000025
25.2	0.000026
26.2	0.000027
27.2	0.000028
28.1	0.000029
29.1	0.000030

The intense color produced with *m*-cresol is qualitatively identical with that obtained with *o*-cresol. In this case, however,

the time of complete development is about 5 minutes although most of the color appears immediately. At first the color is slightly more yellow than the (MO) comparison standard. In the course of 3 minutes the match is perfect and a maximum of color is obtained in 5 minutes. The color fades slowly and becomes pinker. The previously described (MO) standard indicator solution was used for comparisons in the compilation of Table III. This table shows clearly that the color production is directly proportional to the amount of *m*-cresol used.

Estimation of Small Amounts of p-Cresol.

A stock 0.10 per cent solution of *p*-cresol was prepared by dissolving 1.000 gm. of the colorless solid in water and diluting to exactly 1,000 cc. From this the standard solution was prepared by diluting 10 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-cresol.

In this case the color, which is predominantly red, develops to its maximum intensity during the 30 seconds required to mix the contents of the test cylinder. The readings recorded are those obtained *immediately* after the test cylinder was transferred to the colorimeter. The comparison must be made within 2 minutes during which time the color remains of constant and maximum intensity. Then the test liquid, although it changes but little in intensity, rapidly acquires a cloudy appearance which renders further comparisons untrustworthy. This same cloudy appearance is also obtained when the test liquid contains a finely divided precipitate which leads us to believe that an insoluble compound appears, after 2 minutes, although none can be seen with the naked eye.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table IV. As can be seen from the table, the color production is directly proportional to the amount of *p*-cresol used.

Estimation of Small Amounts of p-Oxyphenylacetic Acid.

A stock 1.00 per cent solution of *p*-oxyphenylacetic acid was prepared by dissolving 1.0000 gm. of the colorless solid in water and diluting to 100 cc. This solution was preserved with chloroform.

From it, the standard solution was prepared by diluting 1 cc. to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenylacetic acid.

TABLE IV.

Estimation of Small Amounts of p-Cresol.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Cresol in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
2.5	0.000005
3.0	0.000006
3.5	0.000007
4.0	0.000008
4.5	0.000009
5.0	0.000010
5.5	0.000011
6.0	0.000012
6.5	0.000013
7.0	0.000014
7.5	0.000015
8.0	0.000016
8.5	0.000017
9.0	0.000018
9.5	0.000019
10.0	0.000020
10.5	0.000021
11.0	0.000022
11.5	0.000023
12.0	0.000024
12.5	0.000025
13.0	0.000026
13.5	0.000027
14.0	0.000028
14.5	0.000029
15.0	0.000030

In this case the color, which is predominantly red, develops to its maximum intensity in about 2 minutes. This color of maximum intensity is permanent for about 5 minutes after which it fades slowly and becomes yellow.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table V. It has a color that is slightly more red than that produced by *p*-oxyphenylacetic acid; but the match is so close that accurate comparisons are easily made. As can be seen from Table V the color production is directly proportional to the amount of *p*-oxyphenylacetic acid used.

TABLE V.

Estimation of Small Amounts of p-Oxyphenylacetic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenylacetic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
3.6	0.000010
4.0	0.000011
4.3	0.000012
4.7	0.000013
5.0	0.000014
5.4	0.000015
5.7	0.000016
6.1	0.000017
6.5	0.000018
6.8	0.000019
7.2	0.000020
7.6	0.000021
7.9	0.000022
8.3	0.000023
8.6	0.000024
9.0	0.000025
9.4	0.000026
9.7	0.000027
10.1	0.000028
10.4	0.000029
10.8	0.000030

Estimation of Small Amounts of p-Oxyphenylpropionic Acid.

A stock 1.00 per cent solution of *p*-oxyphenylpropionic acid was prepared by dissolving 1.0000 gm. of the solid in 30 cc. of 0.20 N NaOH and diluting, with water, to exactly 100 cc. Chloroform was added as a preservative. The standard solution was prepared from the stock solution by diluting 1.00 cc. of the latter

to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenylpropionic acid. The color, which is predomi-

TABLE VI.

Estimation of Small Amounts of p-Oxyphenylpropionic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenylpropionic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*gm.*
3.1	0.000010
3.4	0.000011
3.7	0.000012
4.0	0.000013
4.3	0.000014
4.6	0.000015
5.0	0.000016
5.3	0.000017
5.6	0.000018
5.9	0.000019
6.2	0.000020
6.5	0.000021
6.8	0.000022
7.1	0.000023
7.4	0.000024
7.7	0.000025
8.1	0.000026
8.4	0.000027
8.7	0.000028
9.0	0.000029
9.3	0.000030
9.6	0.000031
9.9	0.000032
10.2	0.000033
10.5	0.000034
10.8	0.000035
11.1	0.000036
11.5	0.000037
11.8	0.000038
12.1	0.000039
12.4	0.000040

nantly red, develops to its maximum intensity in about 2 minutes and undergoes no change for from 1 to 3 minutes depending

upon the quantity of *p*-oxyphenylpropionic acid present and the room temperature. This period of color constancy is followed, rather sharply, by a rapid decline in color intensity, a cloudy appearance and a change in tint. The values recorded in Table VI are those obtained just previous to this change.

The previously described (CR) standard indicator solution was used for comparisons in the compilation of Table VI. This table shows that the color production is directly proportional to the amount of *p*-oxyphenylpropionic acid present.

Estimation of Small Amounts of p-Oxyphenyllactic Acid.

A stock 1.00 per cent solution of *p*-oxyphenyllactic acid was prepared by dissolving 1.0000 gm. of the pure solid in 56 cc. of 0.1 N NaOH and diluting with water to exactly 100 cc. Chloroform was added as a preservative. The standard solution was prepared by diluting 1.00 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of *p*-oxyphenyllactic acid.

The color, which is predominantly red, develops to its maximum intensity in about 4 minutes and undergoes no change for about 5 minutes. The (CR) standard, which was used for comparisons in the compilation of Table VII, is slightly more red than the color produced by *p*-oxyphenyllactic acid; but the discrepancy in tint is too slight to interfere with the accuracy of the comparisons. The slowness of the color development, the slight yellow tint, and the stability of the color are characteristics for oxyphenyllactic acid. In this case there is no sharp color change and the liquid does not become cloudy as in the case of oxyphenylpropionic acid.

The tabular values have been carried out to the second place because these figures are obviously more correct than the actual readings which can, of course, be obtained with an accuracy of only one decimal place. Thus for 0.000019 gm. of *p*-oxyphenyllactic acid a reading of either 4.70 or 4.80 mm. is obtained; but the correct reading would be 4.75. This figure (4.75) therefore, appears in the table. As in the case of the other phenols, the color production is directly proportional to the amount of the phenol used.

TABLE VII.

Estimation of Small Amounts of p-Oxyphenyllactic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	*p*-Oxyphenyllactic acid in the test cylinder (total volume 8 cc.) test cylinder set at 20 mm.
mm.	*γm.*
2.5	0.000010
2.75	0.000011
3.0	0.000012
3.25	0.000013
3.5	0.000014
3.75	0.000015
4.0	0.000016
4.25	0.000017
4.5	0.000018
4.75	0.000019
5.0	0.000020
5.25	0.000021
5.5	0.000022
5.75	0.000023
6.0	0.000024
6.25	0.000025
6.5	0.000026
6.75	0.000027
7.0	0.000028
7.25	0.000029
7.5	0.000030
7.75	0.000031
8.0	0.000032
8.25	0.000033
8.5	0.000034
8.75	0.000035
9.0	0.000036
9.25	0.000037
9.5	0.000038
9.75	0.000039
10.0	0.000040
10.25	0.000041
10.5	0.000042
10.75	0.000043
11.0	0.000044
11.25	0.000045
11.5	0.000046
11.75	0.000047
12.0	0.000048
12.25	0.000049
12.5	0.000050

Procedure for the Estimation of Small Amounts of Tyrosine and Tyramine.

Process II.

As has already been pointed out in the introduction, tyrosine and tyramine show an anomalous behavior toward *p*-phenyldiazonium sulfonate in alkaline—sodium carbonate—solutions. When a solution of tyrosine or tyramine is added to the alkaline reagent, a pink color develops promptly, as in the case of the aromatic hydroxy-acids. After about 30 seconds, however, the color changes sharply to yellow and fades. The pink color is too evanescent to compare and the intensities of the yellow colors are not directly proportional to the amount of tyrosine or tyramine used. The procedure that has been found to give satisfactory results with the other phenols, and with imidazoles, cannot be used for the estimation of tyrosine or tyramine. The procedure finally adopted is given below; the reasons for its adoption and an explanation of the chemical reactions involved have already been given in the introduction.

Process I described for the other phenols (see page 246) is followed as far as foot-note 14. The test cylinder is allowed to stand for exactly 5½ minutes from the time that the tyrosine or tyramine solution began to mix with the alkaline reagent. This gives rise to a primary color that is yellow to orange and whose intensity is not directly proportional to the amount of the phenol used.

Sodium hydroxide (2.00 cc. of a 3.0 N solution) is added and the contents of the cylinder thoroughly mixed as before. This gives rise to a marked color intensification with a change of tint toward red, the color still being, however, predominantly yellow.

Exactly 1 minute after the sodium hydroxide solution began to mix with the liquid in the test cylinder, 0.10 cc. of a 20 per cent solution of hydroxylamine hydrochloride[15] is rapidly introduced and the contents of the cylinder are again thoroughly and rapidly mixed. At first there is no change in color. Then suddenly, after a latent period of from 5 to 10 seconds, an intense bluish red color

[15] This was obtained from the Special Chemicals Co., Highland Park, Illinois.

develops. This secondary color develops to its full intensity while the cylinder and contents are being agitated (30 seconds) and changes very little in tint or intensity in half an hour. It is best to introduce the hydroxylamine solution from a rapid delivery 0.10 cc. pipette and to have the liquid well mixed before the secondary color *begins* to develop. The test cylinder is transferred to the right-hand side of the Duboscq colorimeter and set at 25 mm. The left-hand cylinder, which contains the previously described (F–MO) comparison standard, is then adjusted until the two halves of the field are identical in tint and intensity.

The color is easily compared and the determinations are accurate to about 0.5 to 1.5 per cent. If the process is properly carried out, the correction blank (see later) is about 0.30 mm. (F–MO). This amount must be subtracted from the actual reading to obtain the values recorded in Table VIII. We have found that a high laboratory temperature raises this correction blank from 0.10 to 0.20 mm. *The hydroxylamine solution must not be introduced until the sodium hydroxide has been allowed to react with the alkaline reagent for at least 1 minute;* otherwise a colored compound is produced with the hydroxylamine.

The tabular values are accurate for laboratory temperatures ranging from 18 to 25°. At lower temperatures the colors are very slightly less intense and at higher temperatures they are slightly more intense. It is always advisable, before carrying out a determination on an unknown, to run a few determinations on a standard tyrosine solution to be certain that the tabular values are accurate for the existing laboratory conditions.

It is a curious coincidence that the tabular values are identical for both tyrosine and tyramine hydrochloride. The same table may, therefore, be used in the estimation of either.

A stock 1.00 per cent solution of tyrosine was prepared by dissolving 2.0000 gm. of the pure solid in 75 cc. of 1.0 N HCl and diluting with water to 200 cc. From this the standard solution was prepared by diluting 1 cc. to 100 cc. in a volumetric flask. Each cc. of the standard solution contained 0.0001 gm. of tyrosine.

A stock 1.00 per cent solution of tyramine hydrochloride was prepared by dissolving 2.0379 gm. of the 98.14 per cent solid in water and diluting to exactly 200 cc. Chloroform was added as a preservative. The standard solution was prepared by diluting

1.00 cc. of the stock solution to 100 cc. in a volumetric flask. Each cc. of the standard solution contained 0.0001 gm. of tyramine hydrochloride.

TABLE VIII.

Estimation of Small Amounts of Tyrosine and Tyramine Hydrochloride.

Depth of indicator solution (F—MO) required to match the color in the test cylinder.	Tyrosine or tyramine hydrochloride in the test cylinder (total volume 10.1 cc.) test cylinder set at 25 mm.
mm.	*gm.*
4.0	0.000005
4.8	0.000006
5.6	0.000007
6.4	0.000008
7.2	0.000009
8.0	0.000010
8.8	0.000011
9.6	0.000012
10.4	0.000013
11.2	0.000014
12.0	0.000015
12.8	0.000016
13.6	0.000017
14.4	0.000018
15.2	0.000019
16.0	0.000020
16.8	0.000021
17.6	0.000022
18.4	0.000023
19.2	0.000024
20.0	0.000025
20.8	0.000026
21.6	0.000027
22.4	0.000028
23.2	0.000029
24.0	0.000030

The Correction Blank.

When the reagent and alkali are mixed in the absence of a phenol derivative, a very pale yellow color is produced in the course of 5 minutes. This color has an intensity value equivalent to about 0.30 mm. of any of the standard indicator solutions used. This

same amount of color is also produced in the presence of phenol derivatives along with the color produced by the phenol; so the readings obtained are high by about 0.30 mm. in every case. It is necessary, therefore, to subtract 0.30 mm. from the readings obtained before comparison with the tables.

Substances That Do and Do Not Interfere with the Quantitative Determination of Phenols.

The tests for interference were carried out as follows, unless otherwise specified.

Tyrosine (1.00 cc. of the stock 1 per cent solution) was mixed, in a 100 cc. graduated precision cylinder, with quantities of the interfering substances as given below. Water was added to give a total volume of 100 cc. Then 0.10 and 0.20 cc. portions of this liquid were taken for the colorimetric determinations.

Interference with the determination of tyrosine was studied particularly because this determination involves a heretofore unstudied type of reaction. Phenols other than tyrosine and tyramine are determined by a process identical with that used for imidazoles and the interference with that process has already been studied and reported.

Sodium and potassium chloride, sulfate, phosphate, acetate, and citrate in 5 per cent concentrations do not interfere with the colorimetric determination of tyrosine or tyramine.

Ammonium salts interfere very seriously with the determination. When the concentration of ammonium chloride is 5 per cent, so much of a greenish yellow color is produced that the *presence* of tyrosine would not be suspected. Needless to say, tyrosine could not be determined under these conditions. When the concentration of ammonium chloride is 1 per cent, the interference is still considerable because 0.10 cc. of the solution had a color value equal to 9.0 mm. (F–MO) as compared to a normal value of 8.0 mm.; which is a positive error of 12.5 per cent. The color was quite yellow. When the concentration of ammonium chloride is reduced to 0.5 per cent results were obtained that are fairly satisfactory for now 0.10 cc. had a color value equal to 8.2 mm. (F–MO) which is 102.5 per cent of the correct reading. The color was exactly like that of the standard. *Ammonium salts must not be present in concentrations exceeding 0.5 per cent in liquids that are to be examined for tyrosine colorimetrically.*

Amino-acids also interfere seriously with the colorimetric estimation of tyrosine as can be seen from the following data.

Leucine.—A solution was prepared containing 5 cc. of a 1 per cent leucine solution, 0.20 cc. of a 1 per cent tyrosine solution, and sufficient water to give a total volume of 10 cc. Of this solution

0.05 cc. had a color value equivalent to 10.6 mm. (F – MO) and
0.10 " " " " " " " 18.7 " (F – MO).

The color contained far more yellow than the comparison standard. Both of these values are high, the 0.05 cc. reading being 30 per cent high and the 0.10 cc. reading being 15 per cent high. This excess of color is due to a yellow compound that is formed when leucine reacts with *p*-phenyldiazonium sulfonate. In this case the ratio of leucine to tyrosine was 25 to 1.

A second liquid was prepared containing 1.00 cc. of a 1 per cent leucine solution, 0.10 cc. of a 1 per cent tyrosine solution, and sufficient water to give a total volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.4 mm. (F – MO) and
0.20 " " " " " " " 16.8 " (F – MO).

The color obtained contained slightly more yellow than the comparison standard; but the match was very good. These values are 5 per cent higher than they should be. The ratio of leucine to tyrosine in this case was 10 to 1.

These experiments show that *leucine interferes seriously with the colorimetic estimation of tyrosine when the ratio of leucine to tyrosine exceeds 10 to 1.*

Glycine.—A solution was prepared containing 0.30 cc. of a 1 per cent glycine solution and 0.10 cc. of a 1 per cent tyrosine solution in a total aqueous volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F – MO) and
0.20 " " " " " " " 16.0 " (F – MO).

The color produced matched that of the standard perfectly. The values obtained are exactly what they would have been if glycine had not been present. When the ratio of glycine to tyrosine is 3 to 1, the colorimetric estimation of tyrosine is not interfered with.

A second solution was prepared containing 0.50 cc. of a 1 per cent glycine solution and 0.10 cc. of a 1 per cent tyrosine solution in a total aqueous volume of 10 cc. Of this solution

0.10 cc. had a color value equivalent to 8.5 mm. (F − MO) and
0.20 " " " " " " " 17.0 " (F − MO).

The color produced was distinctly yellow and not easy to compare with the (F–MO) comparison standard. The values are too high by 6 per cent. Even at this ratio (5 to 1), glycine interferes seriously with the colorimetric determination of tyrosine. With higher concentrations of glycine the interference becomes so pronounced that a determination of any kind is impossible. The color produced is predominantly yellow so that the color due to tyrosine is masked almost completely.

These experiments show that *glycine interferes seriously with the colorimetric estimation of tyrosine when the ratio of glycine to tyrosine exceeds 5 to 1.* From these experiments it is clear that the direct determination of tyrosine colorimetrically in the phosphotungstate filtrate fraction of a protein is impossible by means of this method *as it now stands.* Tyrosine, excepting for that part which can be separated by crystallization, is always associated with a high percentage of other amino-acids. To estimate tyrosine under these conditions we have either to separate tyrosine, or some derivative into which it can be quantitatively converted, from the bulk of the other amino-acids, or to remove the interfering NH_2 groups of these other amino-acids without destroying the colorimetric properties of tyrosine. Attempts are now being made in this laboratory to modify this method so that it will be applicable to the estimation of tyrosine in proteins.

Hydrogen Peroxide.—A solution was prepared containing 0.01 gm. of tyrosine and 0.20 cc. of a 3 per cent commercial hydrogen peroxide solution in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 10.0 mm. (F − MO)

as compared to a normal value of 16.0 mm. The color contained far more yellow than the comparison standard. Hydrogen peroxide is, therefore, a very potent interfering substance.

In its presence extremely low and entirely unreliable values are obtained. Fortunately, hydrogen peroxide can be readily removed from an aqueous solution by treating it with platinized asbestos. An example of such a treatment is given below under formaldehyde.

Formaldehyde.—A liquid was prepared containing 0.01 gm. of tyrosine and 5 cc. of commercial formaldehyde in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 9.2 mm. (F – MO).

The reading should have been 16.0 mm. The color matched that of the comparison standard very well.

A liquid was now prepared containing 0.01 gm. of tyrosine and 2 cc. of formaldehyde in a total aqueous volume of 100 cc. Of this solution

0.20 cc. had a color value equivalent to 14.4 mm. (F – MO).

In this case the reading was low by 10 per cent.

Finally a solution was prepared containing 0.01 gm. of tyrosine and 1.00 cc. of formaldehyde in a total aqueous volume of 100 cc. of which

0.20 cc. had a color value equivalent to 16.0 mm. (F – MO).

This is the normal reading. In this concentration (1.0 per cent) formaldehyde *does not* interfere with the colorimetric estimation of tyrosine.

It is of interest to note that *low* values are obtained when formaldehyde is present. Formaldehyde does *not* give a colored substance with *p*-phenyldiazonium sulfonate. In this respect this aldehyde is very different from acetaldehyde, acetone, and acetoacetic acid since the latter substances give intensely colored compounds with *p*-phenyldiazonium sulfonate in alkaline solution after treatment with sodium hydroxide and hydroxylamine. *Formaldehyde cannot form an olefine enol,* it cannot give rise to hydrazoxime derivatives; the other three carbonyls can. This may account for their different behaviors.

Since it is conceivable that tyrosine determinations might have to be carried out on liquids containing formaldehyde, we sought

for a method that would destroy formaldehyde without injuring tyrosine. Blank and Finkenbeiner[16] have described a method for the quantitative conversion of formaldehyde into sodium formate by means of hydrogen peroxide. We found that this method destroyed formaldehyde and did not destroy tyrosine; but the hydrogen peroxide left after the reaction had gone to completion made it impossible to determine the tyrosine colorimetrically. We then removed the hydrogen peroxide by means of platinized asbestos and found that the concentration of tyrosine had not changed. The experiment was conducted as follows.

A solution was prepared containing 0.01 gm. of tyrosine, 7 cc. of water, 3 cc. of commercial formaldehyde, and 17 cc. of 3 N NaOH. The substances were mixed in a 100 cc. graduated cylinder. After a reaction period of about 15 minutes, hydrogen peroxide (50 cc. of a 3 per cent commercial preparation) was slowly added to the contents of the cylinder *without cooling*. After the evolution of gas had ceased (15 minutes) 0.30 gm. of a 5 per cent platinized asbestos was added to the liquid. The evolution of oxygen was practically nil after 30 minutes. The liquid was neutralized with 5 N sulfuric acid (which took 3 cc.) and diluted to 100 cc. Of the clear colorless liquid obtained after filtration,

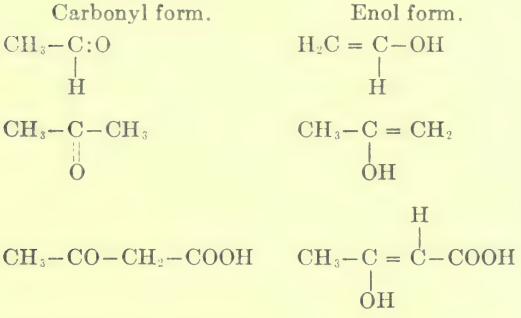
0.10 cc. had a color value equivalent to 8.0 mm. (F – MO) and
0.20 " " " " " " "15.9 " (F – MO)

which is equivalent to 0.01 gm. of tyrosine for the entire test liquid, 100 per cent of the starting material. Formaldehyde and hydrogen peroxide do not destroy tyrosine under the conditions of the above experiment. Both of these interfering substances can be completely removed without interfering with the color-producing properties of tyrosine.

Carbonyl-Enol Interference (Acetaldehyde, Acetone, and Acetoacetic Acid).

These three substances have been classified as carbonyl-enols because they are capable of existing in two tautomeric forms; namely,

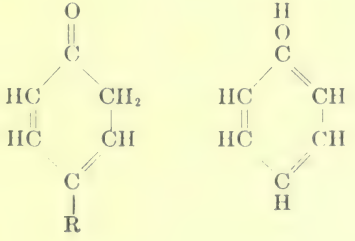
[16] Blank, O., and Finkenbeiner, H., *Ber. chem. Ges.*, 1898–99, xxxi, 2979.



These compounds are particularly potent as interfering agents because they produce a color, under the conditions employed in the estimation of tyrosine, that closely resembles that produced by tyrosine. These colors are so intense that as little as 0.00005 gm. of any of the three substances gives sufficient color to be easily read in a colorimeter.

When the carbonyl derivative is first added to the alkaline reagent a brilliant red color is produced that fades in a few seconds and becomes yellow. The addition of sodium hydroxide, after the initial 5 minute reaction period, enhances this color and changes its tint to bluish red. The subsequent addition of hydroxylamine gives rise to an intense bluish red color that is almost identical with that obtained with tyrosine.

The fact that so simple a substance as acetaldehyde should give a color similar to that obtained with tyrosine would lead one to suspect that this carbonyl-enol tautomerism played a rôle in the color production by tyrosine and tyramine (see introduction for the chemical reactions involved). Phenols can also occur in two tautomeric forms that would properly be called carbonyl and enol, respectively



Acetone is the only member of this group upon which we have carried out any extended experiments. A stock 5 per cent solution of acetone was prepared by diluting 5 gm. of the pure product with water to 100 cc. A standard solution was then prepared by diluting 4 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.002 gm. of acetone. When 0.20 cc. of this standard solution (equivalent to 0.0004 gm. of acetone) was mixed with the alkaline reagent and subsequently treated with sodium hydroxide and hydroxylamine as in the tyrosine determination, a color was produced that was equivalent to about 8.5 mm. (F–MO), the color being slightly redder than that of the comparison standard. It was almost impossible to obtain exactly this value on repetition because the slightest change in the primary reaction time produced a large change in the reading finally obtained. We, therefore, tried several experiments (using 0.20 cc. of the standard acetone solution) in which this reaction time was elongated. With a reaction time of 15 minutes a color value of 29.6 mm. (F–MO) was obtained. With a reaction time of 25 minutes a color value of 31.6 mm. (F–MO) was obtained. Obviously then, the 5 minute reaction period that we found to be ample for the complete development of color in the case of tyrosine and tyramine is too short for acetone. We have made no attempt to change the conditions so that acetone would give a maximum constant color; but this could, no doubt, be done. We wish merely, to call attention to the fact that with 0.0004 gm. of acetone a color value of 29.6 mm. (F–MO) was obtained when the reaction period was 15 minutes; hence as little as 0.00004 gm. of acetone would give a distinctly perceptible color. This method might, therefore, be useful as a qualitative test for traces of acetone even though further experiments might prove that this method could not be made to give readings constant enough for quantitative work.

It seems hardly necessary to say that these carbonyl-enols can never really interfere with the estimation of tyrosine or tyramine because the carbonyl compounds can always be removed by distillation or evaporation.

Glucose.—Glucose and the other sugars containing a free aldehyde or ketone group would be expected to interfere with the colorimetric estimation of tyrosine because of the yellow color

that these compounds give with sodium hydroxide. When 0.10 cc. of a 5 per cent glucose solution is subjected to the treatment used for estimating tyrosine, a very pale yellow primary color is produced which changes to brown with marked intensification on the addition of sodium hydroxide and then becomes redder when hydroxylamine is added. The color finally obtained is still far too yellow to make a comparison with the (F–MO) standard possible. Tyrosine (0.01 gm.) dissolved in 100 cc. of a 5 per cent glucose solution, produces its own color just as it does in the absence of glucose; but the color finally obtained is the sum of the tyrosine and glucose colors. Thus, of this solution

0.10 cc. had a color value equivalent to 10.3 mm. (F – MO) and
0.20 " " " " " " " 19.3 " (F – MO).

The colors obtained in each case contained more yellow than the comparison standard. If these readings are calculated as tyrosine, they are 20 and 13 per cent high, respectively.

Although so high a concentration of glucose would interfere seriously with the colorimetric estimation of tyrosine, lower concentrations are less potent. Thus a tyrosine solution containing 1 per cent of glucose gives a color whose tint and intensity are exactly like those of a solution containing no glucose. *In 1 per cent concentration glucose does not interfere with the estimation of tyrosine.*

Alcohols.—Methyl and ethyl alcohol in 10 per cent concentrations give rise to a color with *p*-phenyldiazonium sulfonate that resembles that produced by tyrosine. The colors are very faint, however, so that a positive interference of about 20 per cent is obtained with 10 per cent concentrations of alcohol. We believe that the pure alcohols do *not* interfere with the colorimetric process and that it is the acetone and acetaldehyde present in the alcohols that produce the color. Ethyl alcohol, that has been freshly distilled over potassium hydroxide augments the color of a tyrosine solution very little even when the alcohol concentration is 10 per cent.

An aqueous tyrosine solution, that has been saturated with amyl alcohol, gives values that are about 5 per cent too high. A chloroform extraction of this liquid *reduces* the reading to about

2 per cent above normal; but does not remove the amyl alcohol completely. Any of these alcohols can, of course, be completely removed by distillation or evaporation; so they can offer no permanent difficulty.

Chloroform, toluene, and ether that has been distilled over sodium, do not interfere with the colorimetric estimation of tyrosine.

Charcoal.—In our earlier work we found that animal or vegetable charcoal adsorbed appreciable quantities of imidazoles and we advised against the use of charcoal in any liquid that was to be tested quantitatively for imidazoles. The adsorption power of charcoal for phenols is far greater than for imidazoles as can be seen from the following data.

5 cc. each of the 1 per cent stock solutions of tyrosine, tyramine, and phenol were separately diluted to 100 cc. and treated with 1 gm. of Pfanstiehl's decolorizing charcoal. After 10 minutes of agitation the liquids were filtered and colorimetric estimations made on the clear filtrates.

Of the tyrosine solution,

0.10 cc. had a color value equivalent to 9.2 mm. (F – MO)

which is equivalent to 0.0115 gm. of tyrosine; hence 0.0385 gm. of tyrosine was adsorbed by 1 gm. of charcoal.

Of the tyramine solution,

0.10 cc. had a color value equivalent to 20.5 mm. (F – MO)

which is equivalent to 0.0256 gm. of tyramine hydrochloride; hence 0.0244 gm. of tyramine hydrochloride was adsorbed by 1 gm. of charcoal.

Of the phenol solution,

1.0 cc. had a color value equivalent to 18.4 mm. (Ph – R)

which is equivalent to 0.0016 gm. of phenol; hence 0.0484 gm. of phenol was adsorbed by 1 gm. of charcoal.

Barium sulfate precipitates do not adsorb phenols from a neutral or acid solution.

SUMMARY.

1. Methods have been devised for the *quantitative colorimetric estimation* of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine.

2. These methods are based upon the well known fact that phenols react with diazonium compounds in alkaline solutions to give colored derivatives. A freshly prepared solution of *p*-phenyldiazonium sulfonate is mixed with a dilute solution of sodium carbonate. A dilute solution of the phenol whose concentration is to be estimated is mixed with the alkaline reagent which gives rise to a primary color that is yellow to red depending upon the character of the phenol.

3. The phenols studied can be divided into three classes.

A. Phenols in which the para position is not occupied by a second substituent.

B. Phenols in which the para position is occupied by a second substituent that does not contain an amino group.

C. Tyrosine and tyramine.

Phenols belonging to Class A (phenol and *o*- and *m*-cresol), couple with great speed and give rise to yellow colors.

Phenols belonging to Class B (*p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids), couple more slowly than those belonging to Class A. The color produced is predominantly red.

Tyrosine and tyramine show an anomalous behavior toward alkaline (Na_2CO_3) *p*-phenyldiazonium sulfonate. An evanescent pink color is produced at first, which fades in 30 seconds to a yellow of inconstant intensity. The simple process employed for the estimation of imidazoles and the other phenols cannot, therefore, be used for the estimation of tyrosine and tyramine. The primary yellow color produced by tyrosine or tyramine is enhanced somewhat by the addition of sodium hydroxide. The colors produced are not directly proportional to the amount of phenol present. If this strongly alkaline liquid is now treated with a small amount of hydroxylamine hydrochloride, a very intense bluish red color is produced whose intensity is directly proportional to the amount of tyrosine or tyramine present (Process II).

4. Tables are given for the direct determination of quantities of these phenols ranging from 0.000001 to 0.00005 gm. The amount of the phenol derivative in any quantity of liquid can then be determined, by multiplication, with an accuracy of from 0.5 to 3 per cent.

5. The alkali salts of the common organic and inorganic acids do not interfere with either of the above two colorimetric processes.

Ammonium salts and amino-acids give an intense yellow color with the process used for the estimation of tyrosine and tyramine (Process II). High values are obtained if these nitrogen compounds are present in sufficient concentration.

Hydrogen peroxide and formaldehyde suppress the color production by tyrosine; hence in the presence of these compounds low values are obtained.

Acetaldehyde, acetone, and acetoacetic acid give rise to a color that is qualitatively identical with that obtained with tyrosine and tyramine. The colors are so intense that the possibility of using this method in the estimation of these carbonyl derivatives suggests itself.

The presence of the ordinary alcohols leads to high readings probably because of the presence of aldehydes or ketones in the alcohols.

1 gm. of vegetable charcoal adsorbs 0.0385 gm. of tyrosine, 0.0244 gm. of tyramine, and 0.0484 gm. of phenol from 100 cc. of an aqueous solution that originally contained 0.05 gm. of the above phenols.

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STUDIES ON PROTEINOGENOUS AMINES.

XV. A QUANTITATIVE METHOD FOR THE SEPARATION AND ESTIMATION OF PHENOLS INCLUDING PHENOL, *o*-, *m*-, AND *p*-CRESOL, *p*-OXYPHENYLACETIC, *p*-OXYPHENYLPROPIONIC, AND *p*-OXYPHENYLLACTIC ACIDS, TYROSINE, AND TYRAMINE.

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A study of the products formed from tyrosine by the action of living cells has always been less difficult than the study of the products formed from histidine because the phenols formed can be easily separated into several well defined groups. Experiments on tyrosine have almost invariably been conducted on a large scale, however, because the products formed have had to be identified and estimated by a process of isolation and purification. A purely chemical method, *applicable to small amounts of material, that would effect a quantitative separation into the maximum possible number of groups and that would permit an accurate determination of the constituents of each group without the necessity for an actual isolation of the constituents*, would be superior to any previously described method.

In the preceding communication,[1] two methods were described by means of which small amounts of phenols can be accurately estimated. In that paper, no claims were made for the applicability of the method to mixtures of phenols. It is the object of this report to show that the methods can also be applied to mixtures of phenols and that the exact quantity of phenol, *o*-, *m*-, and *p*-cresol, *p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids, tyrosine, and tyramine can be rapidly and accurately determined. The method has been found to be

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1922, l, 235.

applicable, without modification, to liquid media in which bacteria have been allowed to metabolize tyrosine in the presence of salts and glycerol or glucose. In its present form the method is not directly applicable, in its entirety, to more complex liquids such as urine or blood. We hope to modify the method so that it can be applied to such liquids. The underlying principles of the method are as follows:

The simple phenols that are apt to occur associated with living matter can be divided into four groups.

A. Those volatile with steam (phenol and *p*-cresol).

B. Those that will pass into ether from an acidified aqueous solution (*p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids).

C. Tyramine, which can be extracted from an alkaline (sodium carbonate) solution by means of amyl alcohol.

D. Tyrosine, which remains after all of the other phenols have been extracted and which can be determined colorimetrically if imidazoles and amino-acids are not present in high concentrations.

The acidified mixture of phenols is first subjected to a distillation under ordinary pressures. The volatile phenols, phenol or *p*-cresol,[2] pass over quantitatively into the distillate where they can be determined colorimetrically if only *one* of them is present. If these two phenols are present together in nearly equal amounts our method will not estimate them because phenol gives a yellow and *p*-cresol a red color with alkaline *p*-phenyldiazonium sulfonate; and these two colors cannot be estimated separately with a Duboscq colorimeter.

The residue obtained after the volatile phenols have been removed by distillation, is transferred to a glass dish, concentrated to a syrupy consistency on the water bath, and diluted to exactly 25 cc. Of this solution 10 cc. are transferred to an extraction bottle and extracted ten times with ether using 20 cc. for each extraction. *p*-Oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids pass quantitatively into the ether. The combined ether extracts are treated with water (25 cc.) and phosphoric acid (5 drops of the 85 per cent acid) after which the ether is

[2] *o*- and *m*-cresol are also quantitatively volatile with steam. We have not included them in our analytical process because they are not apt to occur in biological fluids under ordinary conditions.

removed by distillation, at first under ordinary pressures and then *in vacuo*. The aqueous solution is then diluted to 100 cc. and the concentration of aromatic hydroxy-acids determined colorimetrically.

Our method does not differentiate between the members of the aromatic hydroxy-acid fraction if they are present together in the same solution. Usually, however, only one member of this group will be present under any given condition. It is possible to tell not only *how much* of a given acid is present but also *which one of the acids is present* by the character of the color produced. The color produced by *p*-oxphenylacetic acid *develops to its maximum intensity within 2 minutes and is slightly brown.*

The color produced by *p*-oxyphenylpropionic acid is also fully developed within 2 minutes; but it contains no brown and it undergoes a sharp change after 2 to 3 minutes of color constancy, that is characteristic for *p*-oxyphenylpropionic acid. The color, which has matched that of the (CR) comparison standard perfectly, suddenly takes on a cloudy appearance and a bluish tint that makes further comparisons impossible.

The color produced by *p*-oxyphenyllactic acid *comes up slowly so that 5 minutes are required to give a color of maximum intensity.* The color finally obtained is slightly more *yellow* than that of the (CR) comparison standard. It is also quite stable: *it does not fade perceptibly for 5 minutes after it has reached its maximum intensity.* This color does *not* undergo a change like that of *p*-oxyphenylpropionic acid.

The acid-containing aqueous liquid, which has been freed from volatile phenols by distillation and from aromatic hydroxy-acids by ether extractions, is carefully treated with solid anhydrous sodium carbonate until the liquid stops effervescing. An excess of sodium carbonate (2 gm.) is then added and the alkaline aqueous liquid extracted six times with amyl alcohol, using 20 cc. for each extraction. The amyl alcohol extracts contain tyramine and the alkaline aqueous liquid contains tyrosine.

A quintuple extraction of the amyl alcohol extracts with N H_2SO_4 removes the tyramine completely from the amyl alcohol. Tyramine can then be estimated colorimetrically in the aqueous acid liquid after neutralization and dilution to 100 cc.

The tyrosine fraction, which contains an excess of sodium carbonate, is transferred to a glass dish, treated with an excess of 37 per cent HCl, and concentrated on the water bath. The crystalline residue obtained is transferred with water to a 25 cc. graduated cylinder and diluted to the mark. Tyrosine is then estimated colorimetrically, in this fraction.

Sections I to IV of this report contain a detailed account of the results of experiments on known solutions of phenol derivatives by which the accuracy of the technique of the method for separating the phenols, described in Section V was experimentally established.

EXPERIMENTAL PART.

I. Tyrosine and Tyramine Not Destroyed by the Prolonged Action upon Them of Hot Hydrochloric Acid and Sodium Hydroxide.

A. Hot 20 Per Cent Hydrochloric Acid.

Tyrosine.—Tyrosine (0.1000 gm.) was mixed with 100 cc. of 20 per cent HCl in a 400 cc. round bottomed Pyrex flask. The solution was boiled for 24 hours over an electrically heated sand bath. The resulting pale yellow liquid was evaporated on the water bath in a glass dish. The residue was treated with water and sufficient hydrochloric acid to give a clear solution. This liquid was then transferred to a volumetric flask and diluted to 1,000 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " " 16.0 " (F−MO).

The entire test liquid must, therefore, have contained 0.1000 gm. of tyrosine which is exactly the amount originally introduced.

Continued boiling with 20 per cent hydrochloric acid does not destroy tyrosine to the slightest extent.

An entirely similar experiment was carried out on 0.1000 gm. of tyramine hydrochloride. Of the solution finally obtained

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " " 16.0 " (F−MO).

This, by table[3] is equal to 0.1000 gm. of tyramine hydrochloride for the entire test solution which is 100 per cent of the amount originally introduced.

Tyramine is not injured to the slightest extent by continued boiling with 20 per cent hydrochloric acid.

B. Hot 10 Per Cent Sodium Hydroxide.

Tyrosine.—Tyrosine (0.10 gm., 10 cc. of a 1 per cent solution) was mixed with 10 cc. of a 20 per cent solution of sodium hydroxide. The resulting solution was then heated for 10 hours on the boiling water bath in a small, long necked, round bottomed flask. The resulting colorless liquid was transferred, with water, to a 1,000 cc. volumetric flask, neutralized to litmus paper with 5 N H_2SO_4, and diluted to 1,000 cc. Of this solution

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " " 16.0 " (F−MO),

which for the entire solution represents 0.10 gm. of tyrosine, 100 per cent of the amount originally introduced.

Tyrosine is not injured to the slightest extent when it is heated for 10 hours with 10 per cent sodium hydroxide.

Tyramine.—An entirely similar experiment was carried out on 0.1000 gm. of tyramine hydrochloride. Of the solution finally obtained

0.10 cc. had a color value equivalent to 8.0 mm. (F−MO) and
0.20 " " " " " " " 16.0 " (F−MO)

which, by table, is equivalent to 0.10 gm. of tyramine hydrochloride, 100 per cent of the amount originally introduced.

Tyramine is not injured to the slightest extent when it is heated for 10 hours with 10 per cent sodium hydroxide.

II. Phenol and o-, m-, and p-Cresol are Quantitatively Volatile with Steam.

p-Cresol (0.01 gm., 10 cc. of the stock 0.1 per cent solution) was mixed with 140 cc. of water in a 500 cc. distilling flask. The

[3] Tables for converting colorimetric readings into gm. of phenols are given in the preceding article (*J. Biol. Chem.*, 1922, l, 235).

flask was heated with a gas burner through a small hole in an asbestos gauze. The hot vapors were condensed in a spiral glass condenser, the distillate being collected in a 100 cc. graduated cylinder.

Collections.

1. Total volume of the distillate was 50 cc., of which
0.10 cc. had a color value equivalent to 8.0 mm. (CR).
This, by table, is equivalent to 0.0080 gm. of *p*-cresol, 80 per cent of that originally introduced.
2. Total volume of the distillate was 50 cc. of which
0.40 cc. had a color value equivalent to 6.3 mm. (CR).
This, by table, is equivalent to 0.001575 gm. of *p*-cresol, 15.75 per cent of the amount originally introduced.

At this time, 100 cc. of distilled water were introduced into the distilling flask and the distillation was continued.

3. Total volume of the distillate was 60 cc. of which
1.00 cc. had a color value equivalent to 3.0 mm. (CR).
This, by table, is equivalent to 0.00036 gm. of *p*-cresol, 3.6 per cent of the amount originally introduced.
4. The fourth 50 cc. of distillate had no color value.
The total recovery of *p*-cresol in this case was

First 50 cc. contained	0.0080 gm.
Second 50 " "	0.001575 "
Third 60 " "	0.00036 "
	0.009935 "

which is 99.35 per cent of the amount originally introduced.

A second experiment was now carried out to see if a large quantity of *p*-cresol could be recovered quantitatively. This was a duplicate of Experiment 1 excepting that 0.10 gm. of *p*-cresol was used instead of 0.01 gm.

Collections.

1. Total volume of the distillate was 100 cc. The color produced with 0.10 cc. of this liquid was so intense that a direct comparison was impossible; hence 10 cc. of the distillate were diluted to 100 cc. Of this diluted solution
0.20 cc. had a color value equivalent to 9.4 mm. (CR)
which, by table, is equivalent to 0.094 gm. of *p*-cresol for the entire distillate, 94 per cent of the amount originally introduced.

2. Total volume of the distillate was 100 cc. of which
0.20 cc. had a color value equivalent to 6.3 mm. (CR).
This, by table, is equal to 0.0063 gm. of *p*-cresol for the entire distillate, 6.3 per cent of the amount originally introduced.
3. Total volume of the distillate was 50 cc. of which
1.00 cc. had a color value equivalent to 7.2 mm. (CR).
This, by table, is equal to 0.000072 gm. of *p*-cresol, 0.072 per cent of the amount originally introduced.
4. Total volume of the distillate was 50 cc. of which
1.00 cc. had a color value equivalent to 3.5 mm. (CR).
This is equal to 0.000035 gm. of *p*-cresol, 0.035 per cent of the amount originally introduced.
5. The fifth 50 cc. of distillate had no color value.

The total recovery in this case was

First	100 cc. contained	0.094000	gm.
Second	100 " "	0.0063	"
Third	50 " "	0.000072	"
Fourth	50 " "	0.000035	"
		0.100407	" of *p*-cresol

which is 100.4 per cent of the amount originally introduced.

Entirely similar experiments were conducted on solutions of phenol, and *o*- and *m*-cresol. In every case between 99 and 100.5 per cent of the phenol was recovered and accounted for, colorimetrically, in the distillate.

Phenol, o-, m-, and p-cresol are completely volatile with steam and they can be estimated quantitatively in the distillates.

III. The Aromatic Hydroxy-Acids Can Be Quantitatively Extracted from an Acidified Aqueous Solution with Ether.

When an acidified aqueous solution containing any of the aromatic hydroxy-acids is extracted ten times with redistilled ether, the aromatic hydroxy-acids pass quantitatively into the ether because *the aqueous liquid no longer gives the slightest color with Pauly's reagent.*[4] If the ether, which must surely contain all of the aromatic hydroxy-acids, is now removed by distillation, and the residue diluted with water to a definite volume, *a colorimetric determination by the usual process either fails to reveal the presence of any phenol or gives values that are far below the theoretical.*

[4] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

We thought, at first, that the aromatic hydroxy-acids might be slightly volatile with steam or ether vapor; but we soon proved that this was *not* true. The other possibility was that the ether contained some imperfectly volatile substances that prevented the phenol from combining with *p*-phenyldiazonium sulfonate in alkaline solution. We surmised that the interfering substance was an oxidation product of the ether, perhaps peroxide in character. To remove this, we agitated a sample of redistilled ether with alkaline permanganate until the permanganate had been decolorized, and redistilled the ether layer. An extraction of the aromatic hydroxy-acids with this *freshly prepared* ether was then carried out. Theoretical values were always obtained with these ether extracts. If, however, the ether was not used for a day or two, low values were again obtained. To avoid the necessity of having to prepare a fresh supply of ether each day, we modified the usual colorimetric procedure as follows.

(1-X) cc. of water and X cc. of the aromatic hydroxy-acid-containing liquid were treated, for 2 minutes, with 2 cc. of the nitrous acid containing *p*-phenyldiazonium sulfonate reagent. The 1.1 per cent sodium carbonate solution (5 cc.) was then added. This inverse process, which is similar to the process usually used for the qualitative determination of phenols or imidazoles, gave theoretical values both with pure solutions of aromatic hydroxy-acids and with ethereal extracts *even when the ether had not been previously treated with permanganate.* Obviously, then, the free nitrous acid present in the reagent modified the interfering substances so that they no longer prevented the combination between the phenol and the diazonium salt. Fortunately, the nitrous acid did not react with the aromatic hydroxy-acids in such a way as to prevent their coupling with the diazonium salt. *This inverse process should always be used in the estimation of aromatic hydroxy-acids.*

The following experiment will illustrate the method used in the extraction and estimation of the aromatic hydroxy-acids.

p-Oxyphenylpropionic acid (1.00 cc. of the 1 per cent stock solution was mixed in a 35 cc. extraction bottle[5] with 0.2 cc. of 95 per

[5] Any 35 cc. narrow mouthed bottle with a carefully fitted glass stopper will answer the purpose.

cent H_2SO_4 and 9 cc. of water. 20 cc. of specially prepared ether,[6] measured by graduate, were introduced into the bottle, the glass stopper inserted and the liquids vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube and centrifuged for from 1 to 2 minutes. This gave a sharp separation into two layers.

Separation of Ether.—The ether layer was separated from the aqueous layer by means of a device similar to that shown in a previous article.[7] The ether, instead of being drawn into a Squibb funnel, is drawn into a 700 cc. round bottomed flask. Capillary F is, at first, carefully immersed *just below the surface of the ether layer* because the vaporization of the ether as it comes in contact with the large surface of the warm flask usually produces enough pressure to eject some ether back into the extraction bottle which would stir up the aqueous layer if capillary F was too deeply immersed. After the first momentary back pressure, however, capillary F can be gradually lowered until all but a thin film of ether has been drawn into the receiving flask.

The ether not only extracts the aromatic hydroxy-acids but it also reduces the volume of the aqueous layer. It is necessary, therefore, to add sufficient water after each extraction to reestablish the initial volume of 10 cc. It is best to mark the extraction bottle with a carborundum pencil at a level corresponding to a volume of approximately 10 cc.

The above process was repeated nine times so that a total of ten extractions was made.

The aqueous acid liquid gave no Pauly reaction. The ether extracts were then treated with 25 cc. of water and 5 drops of 85 per cent phosphoric acid. The mixture was agitated and subjected to a distillation at first under atmospheric pressure and then *in vacuo* until the ether had been removed entirely. An ebullition tube was *not* used and the distillation was *not* contin-

[6] Commercial ether (800 cc.) is agitated with 50 cc. of alkaline permanganate, such as is used in amino nitrogen determinations by the Van Slyke process, and 50 cc. of water in a 2,000 cc. separatory funnel. After the permanganate has been reduced to MnO_2, the ether layer is poured off and distilled. The redistilled ether so obtained is then ready to use for extractions.

[7] See Fig. 1, Studies on proteinogenous amines. III (Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 526.

ued until all of the water had passed over. The aqueous liquid left in the flask was carefully transferred with water to a 100 cc. graduated precision cylinder and diluted to 100 cc. Of this liquid, 0.10 cc. was mixed with 0.90 cc. of water and 2 cc. of reagent in the right-hand cylinder of the Duboscq colorimeter. After 2 minutes, 5 cc. of the 1.1 per cent sodium carbonate solution were added. The cylinder was transferred to the colorimeter set at 20 mm. and the color compared in the usual manner with that of the (CR) comparison standard. A reading of 3.1 mm. (CR) was obtained. A 0.20 cc. portion of the liquid had a color value of 6.2 mm. This is equal to 0.01 gm. of *p*-oxyphenylpropionic acid, 100 per cent of the amount originally introduced.

Entirely similar experiments were carried out on solutions of *p*-oxyphenylacetic and *p*-oxyphenyllactic acids. 100 per cent recoveries were obtained in every case.

The aromatic hydroxy-acids are quantitatively extracted from an acidified aqueous solution with ether.

IV. Tyramine Quantitatively Separated from Tyrosine by Means of Amyl Alcohol.

A. When a Small Amount of Tyrosine Is Mixed with a Large Amount of Tyramine.

Tyrosine (0.20 cc. of the 1 per cent solution), tyramine (5 cc. of the 1 per cent solution), 0.20 cc. of 95 per cent H_2SO_4, and 5 cc. of water were mixed in a 35 cc. extraction bottle. Anhydrous sodium carbonate was added carefully until the liquid no longer effervesced. Then 2.00 gm. of the carbonate were added and the liquid was agitated until the solid had dissolved. Redistilled amyl alcohol (20 cc., measured by graduate) was introduced into the bottle, the glass stopper inserted, and the liquids were vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube and centrifuged for about 2 minutes. This gave a sharp separation into two layers.

Separation of Amyl Alcohol.—The amyl alcohol layer was separated from the aqueous alkaline layer by means of the device and technique described in a previous article.[7]

The extraction was repeated five times so that a total of 120 cc. of amyl alcohol was used. As in the case of the previously

described ether extractions, the amyl alcohol extracts not only the tyramine but it also markedly reduces the volume of the aqueous layer. It is necessary, therefore, to add sufficient water after each extraction to reestablish the initial volume of 10 cc.

Removal of Tyramine from Amyl Alcohol.—The combined amyl alcohol extracts were extracted five times, in the same Squibb funnel, with 1.0 N H_2SO_4 using 20 cc. for the first and 10 cc. for each of the remaining four extracts. The sulfuric acid extracts were collected in a 100 cc. glass-stoppered precision cylinder and neutralized to litmus paper with 40 per cent sodium hydroxide. The solution was then rendered very faintly acid by adding a few drops of 1.0 N H_2SO_4, transferring to a glass dish, and evaporating on the water bath to remove the amyl alcohol. The crystalline residue was dissolved in water, transferred to a 100 cc. graduated precision cylinder, and diluted to 100 cc. Of the solution so obtained 0.10 cc. had such a high color value that accurate comparisons were impossible; hence 10 cc. of it were diluted with water to 50 cc. Of this diluted solution

0.10 cc. had a color value of 8.0 mm. (F−MO) and
0.20 " " " " " " 16.0 " (F−MO).

This, by table, is equal to 0.05 gm. of tyramine hydrochloride for the entire *original* test solution which is 100 per cent of the amount originally introduced.

The Alkaline Aqueous Liquid (Tyrosine Fraction).—The alkaline aqueous liquid was transferred with water to a glass dish. The dish was covered with a watch-glass. An excess of 37 per cent HCl was added and the liquid concentrated on the water bath. The crystalline residue was dissolved in water with the aid of a few drops of 37 per cent HCl, and diluted to exactly 100 cc. Of this solution

0.50 cc. had a color value equivalent to 8.0 mm. (F−MO) and
1.00 " " " " " " " 16.0 " (F−MO)

which, by table, is equivalent to 0.002 gm. of tyrosine, 100 per cent of the amount originally introduced.

Tyramine is quantitatively extracted from an alkaline (sodium carbonate) aqueous solution by amyl alcohol. Tyrosine, when present in small amounts, does not pass into amyl alcohol from such an alkaline aqueous solution.

B. When a Large Amount of Tyrosine Is Mixed with a Small Amount of Tyramine.

Tyrosine (5.00 cc. of the stock 1 per cent solution), tyramine (0.20 cc. of the stock 1 per cent solution), 0.20 cc. of 95 per cent sulfuric acid, and 5 cc. of water, were mixed in a 35 cc. extraction bottle. Anhydrous sodium carbonate was added carefully until the liquid no longer effervesced. Then 2.00 gm. of the carbonate were added and the mixture agitated until the solid had dissolved. This liquid was then extracted six times with amyl alcohol as described in Section IV, Part A.

Alkaline Aqueous Liquid (Tyrosine Fraction M).—This was treated as described in Section IV, Part A. The solution was finally diluted to 500 cc. of which

0.10 cc. had a color value equivalent to 7.5 mm. (F−MO) and
0.20 " " " " " " " 15.0 " (F−MO)

which, by table, is equivalent to 0.0469 gm. of tyrosine. Since 0.0500 gm. of tyrosine had been originally introduced, *0.0031 gm. of tyrosine must have passed into the amyl alcohol.*

First Amyl Alcohol Extract.—The combined amyl alcohol extracts were extracted with N H_2SO_4 as described in Section IV, Part A. It was necessary in this case, as it will be in most cases, to remove the sulfuric acid with barium hydroxide to avoid the accumulation of a large amount of salts which would interfere with the subsequent treatment. The combined acid extracts were transferred to a 250 cc. Pyrex flask and heated on the water bath. Barium hydroxide (9.0 gm.) was dissolved in 50 cc. of hot water. The resulting solution was added slowly to the acid liquid. The faintly *acid* mixture so obtained was digested on the water bath for 2 hours and filtered through a hard folded filter. The paper and contents were thoroughly washed with hot water. The filtrate and washings were collected in a glass dish, exactly neutralized with sodium hydroxide, and evaporated on the water bath. The solid residue so obtained was transferred with the aid of a few drops of 5 N H_2SO_4 and 10 cc. of water to a 35 cc. extraction bottle. The liquid was treated with 2.00 gm. of anhydrous sodium carbonate. This alkaline solution was then extracted with amyl alcohol in the usual manner which again divides the

material into two fractions; the alkaline aqueous liquid II, which should contain the tyrosine that passed into amyl alcohol at the time of the first extraction, and the purified tyramine fraction which should be free from tyrosine.

Alkaline Aqueous Liquid II (Tyrosine Fraction II).—This was acidified, evaporated, and finally diluted to 100 cc. as in the case of the main tyrosine fraction. Of this solution,

0.50 cc. had a color value equivalent to 11.2 mm. (F−MO) and
1.00 " " " " " " " 22.4 " (F−MO)

which, by table, is equivalent to 0.0028 gm. of tyrosine. Since 0.0469 gm. of tyrosine was recovered in the first, main tyrosine fraction, a total of 0.0497 gm. of tyrosine was accounted for. This is 99.4 per cent of the amount originally introduced.

Purified Tyramine Fraction.—This was extracted with N H_2SO_4 as previously described. The acid was nearly neutralized with 40 per cent sodium hydroxide. The resulting *faintly acid* solution was transferred to a glass dish and evaporated on the water bath. The residue was transferred, with water, to a precision cylinder and diluted to 100 cc. Of this solution,

0.50 cc. had a color value equivalent to 7.9 mm. (F−MO) and
1.00 " " " " " " " 15.8 " (F−MO).

This, by table, is equal to 0.001975 gm. of tyramine hydrochloride, 98.75 per cent of the amount originally introduced.

When the concentration of tyrosine is high, a small amount of it passes into amyl alcohol from an alkaline (sodium carbonate) solution. To free the tyramine fraction from this small admixture of tyrosine it is necessary to conduct a second amyl alcohol extraction on the first tyramine fraction. This slightly longer process with its double amyl alcohol extraction is to be recommended because it makes the determination of tyramine certain and reliable. In most cases it is probably advisable to remove the excess of sulfuric acid from the *final* tyramine fraction with baryta because such solutions would then be practically free from salts and ready for physiological experiments.

V. Separation of Phenols into Four Fractions: Volatile Phenols Aromatic Hydroxy-Acids, Tyramine, and Tyrosine. The Accurate Determination of One Member of Each Fraction.

The method outlined below is primarily intended to be used in bacterial metabolism studies on tyrosine. We have carried out a sufficient number of such metabolism experiments to be certain that the method gives accurate results. We hope to report these experiments in the near future. Some of the steps in the following experiment were taken, not because they were necessary in this case, but because they were necessary in the metabolism experiments.

A solution containing the following was prepared from the stock solutions; 10 cc. of 1 per cent tyrosine, 4 cc. of 1 per cent tyramine hydrochloride, 4 cc. of 1 per cent *p*-oxyphenyllactic acid, 2 cc. of 1 per cent phenol, 80 cc. of water, and 100 cc. of Nutritive Medium 3.[8]

Filtration.—The clear liquid was forced through a Mandler filter.[9] The flask and filter were washed free from phenols with 200 cc. of water. The filtrate was then treated with 0.50 cc. of 95 per cent H_2SO_4.

Estimation of Volatile Phenols (Phenol).—The filtrate and washings were transferred, with water, to a 1,000 cc. long necked, round bottomed, Pyrex flask. The flask was heated with a gas burner through a small hole in an asbestos gauze. The hot vapors were condensed in a spiral condenser, the distillate being collected in

[8] Nutritive Medium 3 contains:

NH_4Cl	4.00 gm.
KNO_3	2.00 "
KH_2PO_4	8.00 "
NaCl	16.00 "
Na_2SO_4	0.04 "
$NaHCO_3$	8.00 "
$CaCl_2$	0.20 "
Glycerol	80.00 cc.

in a total aqueous volume of 2,000 cc. Koessler, K. K., and Hanke, M. T. *J. Biol. Chem.*, 1919, xxxix, 579.

[9] In bacterial metabolism studies, the hydrogen ion concentration is determined, colorimetrically, on 1 cc. of this filtrate before it is diluted with wash water.

a 250 cc. graduated cylinder. Exactly 200 cc. of distillate were collected of which

0.10 cc. had a color value equivalent to 9.6 mm. (Ph−R) and
0.20 " " " " " " " 19.2 " (Ph−R).

This, by table, is equivalent to 0.01666 gm. of phenol for the entire 200 cc. of test liquid.

The distillation was continued until about 125 cc. of distillate had been collected. This was diluted to exactly 200 cc. Of this solution

0.20 cc. had a color value equivalent to 4.0 mm. (Ph−R) and
0.40 " " " " " " " 8.0 " (Ph−R)

which, by table, is equivalent to 0.0034 gm. of phenol for the entire test solution. In all, 0.02006 gm. of phenol was obtained which is 100.3 per cent of the amount originally introduced. The colors obtained were, in every case, exactly like that produced by pure phenol; hence none of the other phenols volatilized.

Estimation of Aromatic Hydroxy-Acids (p-Oxyphenyllactic Acid).—The liquid left in the flask was carefully transferred, with water, to a glass dish and concentrated on the water bath. The pale yellow syrup was transferred, with water, to a 25 cc. precision cylinder and diluted to exactly 25 cc. We will refer to this as the *test liquid.*

Of this acid test liquid, exactly 10 cc. (measured by pipette) were transferred to a 35 cc. extraction bottle and extracted ten times with specially prepared ether as described in Section III of this paper.

The ether extracts were then treated as described in Section III. The solution finally obtained (volume 100 cc.) was examined colorimetrically for *p*-oxyphenyllactic acid.

0.10 cc. had a color value equivalent to 4.0 mm. (CR)
0.20 " " " " " " " 8.0 " (CR)
0.30 " " " " " " " 12.0 " (CR).

The colors were exactly like that obtained with a pure solution of *p*-oxyphenyllactic acid. This, by table, is equivalent to 0.0400 gm. of *p*-oxyphenyllactic acid *for the entire 25 cc. of test liquid*, which is 100 per cent of the amount originally introduced.

Separation of Tyramine from Tyrosine.—The acid liquid left in the extraction bottle, equivalent to 10 cc. of the test liquid, was carefully treated with anhydrous sodium carbonate until the liquid no longer effervesced. Then 2 gm. of the carbonate were added. The mixture was warmed and agitated until the solid had passed into solution. This solution was then extracted six times with amyl alcohol as described in Section IV of this paper.

The Alkaline Aqueous Liquid (Tyrosine Fraction M).—The alkaline liquid left in the extraction bottle was transferred to a glass dish with 100 cc. of water. The liquid was concentrated on the water bath to a volume of about 50 cc., which removed the ammonia completely. The liquid was then treated with 3.5 cc. of 37 per cent HCl, precautions being taken to prevent loss of the solution through spattering. The strongly acid liquid was concentrated on the water bath. The crystalline residue was dissolved in water, with the aid of a few drops of 37 per cent HCl, transferred to a graduated precision cylinder, and diluted to 25 cc. This is the main tyrosine fraction M.

Of this solution 5 cc. (measured by pipette) were diluted to 80 cc. Of this diluted solution

0.10 cc. had a color value equivalent to 7.3 mm. (F−MO) and
0.20 " " " " " " " 14.6 " (F−MO)

which, by table, is equivalent to 0.0912 gm. of tyrosine for the entire original test liquid, 91.2 per cent of the amount originally introduced. As one would expect from Experiment B, Section IV, some tyrosine passed into the amyl alcohol from which it will be recovered when the second extraction is carried out (see below).

Amino Nitrogen Determination on Tyrosine Fraction M.—The above tyrosine fraction M (5 cc.) was subjected to an amino nitrogen determination by the Van Slyke method. 1.07 cc. of N_2 were obtained at 25° and 747 mm. which is equal to 0.00729 gm. of nitrogen for the entire test liquid. Tyrosine always gives off about 2 per cent more gas by the Van Slyke process than it should theoretically. We have found this to be invariably true and Van Slyke[10] gives figures that are in perfect agreement with

[10] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 193.

this statement. This nitrogen figure must, therefore, be reduced by 2 per cent before the tyrosine value is calculated. The corrected nitrogen figure is 0.00715 gm.; which is equivalent to 0.00925 gm. of tyrosine, 92.5 per cent of the amount originally introduced. The check between the tyrosine values obtained by these two methods is good.

We wish again to call attention to the fact that this fraction *does not* contain *all* of the tyrosine. A second fraction is obtained later when the first tyramine fraction is reextracted with amyl alcohol. The second tyrosine fraction so obtained usually contains about 0.003 gm. of tyrosine which is too little to estimate by the amino nitrogen method but which is easily determined colorimetrically.

First Amyl Alcohol Extract (Tyramine Fraction I).—This fraction contains all the tyramine together with a small amount of tyrosine. A quantitative separation can be effected by carrying out a second extraction with amyl alcohol in alkaline solution.

The amyl alcohol was, therefore, extracted with N H_2SO_4, the acid removed with baryta, the resulting liquid made alkaline with sodium carbonate and reextracted with amyl alcohol as described in Section IV, Part B.

Second Alkaline Aqueous Liquid (Tyrosine Fraction II).—This was acidified, evaporated, and finally diluted to 100 cc. as described in Section IV, Part B. In this case the ammonia was not removed by evaporation because an amino nitrogen determination is not to be carried out. Of the solution so obtained

0.20 cc. had a color value equivalent to 4.8 mm. (F—MO) and
0.40 " " " " " " " 9.6 " (F—MO)

which, by table, is equal to 0.0075 gm. of tyrosine for the *entire original test liquid*. Since 0.0912 gm. of tyrosine was recovered in the main tyrosine fraction M, a total of 0.0987 gm. of tyrosine was accounted for, which is 98.7 per cent of the amount originally introduced.

Purified Tyramine Fraction (Second Amyl Alcohol Extract).—This was extracted six times with N H_2SO_4 as previously described. The acid was nearly neutralized with baryta, the barium sulfate removed by filtration, the filtrate concentrated on the water bath in a glass dish, and the residue dissolved in water and diluted to

exactly 100 cc. as described in Section IV, Part B.[11] Of this solution

0.10 cc. had a color value equivalent to 12.8 mm. (F−MO)

which, by table, is equal to 0.0400 gm. of tyramine hydrochloride, 100 per cent of the amount originally introduced.

SUMMARY.

This paper contains the description of a method by means of which volatile phenols, aromatic hydroxy-acids, tyramine, and tyrosine can be quantitatively separated and estimated. The phenols are determined by a colorimetric process described in the preceding paper. Volatile phenols—phenol, *o*-, *m*-, and *p*-cresol—are distilled off and estimated in the distillate. Aromatic hydroxy-acids—*p*-oxyphenylacetic, *p*-oxyphenylpropionic, and *p*-oxyphenyllactic acids—are extracted with ether from the acidified aqueous liquid which has been freed from volatile phenols by distillation. The aromatic hydroxy-acids are estimated in the ether extracts. The remaining liquid, which contains all of the tyramine and tyrosine, is made alkaline with sodium carbonate and freed from tyramine by extraction with amyl alcohol. Tyramine is then determined in the amyl alcohol extract; tyrosine is determined in the alkaline aqueous liquid. The separations are quantitative and the colorimetric determinations are accurate to 0.5 to 1.5 per cent.

[11] It is necessary to remove the excess of H_2SO_4 with baryta only if physiological or isolation experiments are to be carried out on this fraction. In other cases it is simpler to neutralize with 40 per cent NaOH as described in Section IV of this report.

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EXPERIMENTAL STUDIES ON INFLAMMATION.

I. The Influence of Chemicals upon the Chemotaxis of Leucocytes in Vitro.

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Inflammatory processes usually, if not always, are the result of reaction to chemical substances. Some substances cause severe reactions when in very low concentrations, while others, even in high concentrations, cause little or no inflammation. As far as we can learn, no systematic study has ever been made to determine what chemical, physical, or physiochemical properties a substance must have in order that it may incite inflammation, or that it may be unable to incite inflammation. Can definite laws be discerned governing the capacity of chemical substances to stimulate inflammatory reactions? Is it possible to predict from the chemical or physical properties of a substance its capacity for inflammatory stimulation? Such information, if it can be obtained, should be of great value in chemotherapeutic work, since it might be applied to reducing the inflammatory reaction produced by drugs that must be injected into the tissues, and hence this study was undertaken at the suggestion of Dr. H. Gideon Wells as part of the program of chemotherapeutic investigation that is being conducted in the Otho S. A. Sprague Memorial Institute.

In studying this problem it is necessary to consider the several components of inflammatory reaction; namely, vascular changes, migration of cells and fluids, cell injury, and cell proliferation. In this paper will be considered only one phase of the work, the influence of chemicals upon the chemotaxis of leucocytes *in vitro*, a subject which has already received not a little investigation.

Most of the literature on this subject has been fully reviewed by Wells,[1] who has discussed the subject from the earliest studies of Engelmann who worked with protozoa in 1881, up to the time of Jacques Loeb, who worked with chemical tropisms. This may be briefly summarized in the following lists of the substances supposedly positive, negative, and neutral as chemotactic agents for leucocytes.

Various authors have stated that the strongly positively chemotactic substances are: sterilized and living cultures of bacteria, whether pathogenic or non-pathogenic; papayotin or papain (in rabbits) (Gabritchevsky[2]); carbolglycerol extracts of different viscera and tissues; egg albumin, gelatin, peptone, and alkali albuminate; metallic copper, iron, mercury, and their salts;[3] and substances soluble in lipoids in extreme dilution (Hamburger[4]).

Among the supposedly negatively chemotactic substances the following have been mentioned by various authors: concentrated solutions of sodium and potassium salts; lactic acid in all concentrations; quinine (0.5 per cent); alcohol (10 per cent); chloroform, in watery solution; jequirity (2 per cent, passed through a Chamberland filter); glycerol (10 to 1 per cent); bile; *B. choleræ gallinarum* (Gabritchevsky); mercury perchloride, cholesterol, arsenic, antipyrine, acetic acid; concentrated solutions of calcium, sodium, and potassium salts; alcohol in over 10 per cent concentration, chloroform, bile, glycerol in concentrations of 1 to 10 per cent; and atophan, or phenylcinchonic acid.

Among the neutral or indifferent agents have been mentioned water; calcium and sodium salts in concentrations of 0.1 to 1 per cent; phenol; phlorhizin; papayotin (in frogs); glycogen; peptone; blood; hemoglobin; uric acid up to 2 per cent; and bouillon.

These are only tentative statements, for many contradictory results have been obtained, and it is almost impossible to avoid some experimental errors.

The most important of the later work has been done by Hamburger, and his coworkers, on phagocytosis. This bears a close relation to chemotaxis and must be considered at the same time as chemotaxis. Hamburger[4] has shown that "calcium *per se*," as a substance causes a great rise in phagocytosis; it seems to be the only substance acting in this way, apparently aiding chemotaxis. He has investigated this problem from two view-points, *in vivo* and *in vitro* work both being done. In the *in vitro* work, horse leucocytes from citrated serum were transferred to different media and carbon particles added. The

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[1] Wells, H. G., Chemical pathology, Philadelphia, 4th edition, 1920, Chapter XI.

[2] Gabritchevsky, G., *Ann. Inst. Pasteur*, 1890, iv, 346.

[3] Bloch, G., *Centr. allg. Path. u. path. Anat.*, 1896, vii, 785.

[4] Hamburger, H. J., Physikalisch-chemische Untersuchungen über Phagozyten. Ihre Bedeutung von allgemein biologischem und pathologischem Gesichtspunkt, Wiesbaden, 1912.

percentage of these particles taken up by the leucocytes was used as a measure of the degree of phagocytosis. The *in vivo* work was done by injecting calcium-free and calcium-containing cultures of bacteria (*B. coli*) under the skin in capillary tubes, and then comparing the size of the resulting leucocyte columns in the calcium-free and calcium-containing cultures. Besides the work on calcium, Hamburger has tested many organic and inorganic compounds. He found that, under the conditions of his experiments, hemoglobin had no bad effects on phagocytic activity no matter what concentrations were employed. Collargol and other colloidal silver compounds had no effect up to 0.02 per cent, but above that concentration these compounds were injurious and the injury increased in proportion to the concentration. Quinine was injurious to leucocytes, seeming to act upon the cells themselves. Of the halogens, iodine is more poisonous than bromine; fluorine is a strong poison; iodoform increases phagocytosis; mercury, strontium, and barium are indifferent. All fat solvents in small amounts increase phagocytosis; those used were chloroform, ether, chloral, benzene, camphor, some fatty acids, and turpentine. Citrate and sulfite ions act as a poison, preventing any phagocytosis. Oxygen acts like apnea, preventing phagocytosis; carbon dioxide increases phagocytosis. Potassium cyanide has about the same action as carbon dioxide, an increase followed by a decided decrease in phagocytic activities, and then death of the cells.

Method.

In my work, Wright's method has been employed.[5] Equal volumes of blood must be disposed over equal areas of surface, and come into contact with equal concentrations of the desired reagent throughout. This may be done in the following ways: The first method is to paint hot paraffin containing the desired reagent upon glass slides until 1 mm. thick, then punch circles in the plastic paraffin with a sharp cork-borer, and prize out the discs and clean the glass floor with xylol. The second and preferable method is to use agar in the place of paraffin. Here different concentrations of the drugs desired are mixed with 2 per cent salt-free water solution of agar, then cast into tablets 1 mm. thick, in molds constructed of a pair of glass slides held apart at the ends by pieces of slides 1 mm. thick. The slide which forms the lid of the mold is pushed aside, while the agar is poured in, then brought into position. When this melted agar has set the lateral supports and the lid are removed and circles are cut in the agar with a sharp cork-borer, the discs removed,

[5] Wright, A. E., *Lancet*, 1918, i, 129.

and the cells thus obtained placed upon slabs of 2 per cent salt-free water solution of agar, or upon slabs containing the same concentration of the reagent as contained in the cell. In this way, a window is formed, with the floor and sides of agar containing the desired concentration of the reagent. Two different forms are used. In one, the floor consists of 2 per cent salt-free water solution of agar, the sides of the 2 per cent solution of agar containing the desired reagent. In the other, the floor and sides of the cell contain the same concentras of the substances to be tested. These diff erent cells are then placed upon a glass slide so that each slide contains from eight to twelve cells, each one of which has a different concentration of the reagent. On each slide there is a control cell, the floor and sides being made of the 2 per cent salt-free water solution of agar.

The slides containing the agar slabs were kept in moist chambers made of Petri dishes whose bottom and sides were lined with moist cotton, so that no water would be lost by evaporation from the agar, thus changing the concentrations. The Petri dishes were kept in warm water, the slides thus being heated to 37°C. and kept at this heat so that the temperature would be the same in each window when the blood was run in and all the time after pouring. Equal amounts of blood were then run into the cells thus made. A capillary pipette was filled with blood as soon as the vein was cut, and 2 drops of this uncoagulated blood were allowed to drop into each window immediately so that there would be no chance of clotting. All the windows were filled at one time so that there would be no differences in the blood itself to be taken into consideration. The dishes containing the blood-filled windows on the slabs of agar were put in the incubator and kept at 37°C. for 45 minutes. After incubation, the clots of blood were washed off with warm sodium chloride solution, 0.9 per cent sodium chloride in distilled water, kept at 37°. If the clots were extremely adherent to the sides, the edges were loosened by touching them gently with a probe, or lifted out with fine forceps. A solution of saturated mercuric chloride could then be poured into the windows to fix the leucocytes if desired. The number of leucocytes adhering to the sides and the floor were then counted under the low power (No. 3) of the microscope.

In this work repeated duplicate tests were made with the blood of the same animal. The number of cells migrating into the agar was always the same within the limits of experimental error, the difference never being more than 25 to 50 cells out of a total of 2,000 to 2,500. The same percentage of the total number of cells always adhered to the control slide; *i.e.*, about 4 per cent. The exact number varied with the degree of leucocytosis but the relative number was always approximately the same.

From 0.0001 to 10 per cent molecular concentrations of the different salts were employed, so that the range of chemotaxis might be studied in relation to the per cent of the salt itself, as well as the positive and negative ions composing the salt, as, for example, NaCl as a salt and Na as an ion as compared with Na in Na_2CO_3, or Na_2S_4O, and Cl as an ion as compared with Cl in KCl or $CaCl_2$, etc. In some cases a high concentration showed a marked negative chemotaxis, while a low concentration showed a positive one. Whenever possible, neutral or nearly neutral substances were used, so that the reaction would not interfere with the chemotaxis. If, however, the chemical had an acid or alkaline reaction strong enough to give irregular and peculiar results, sodium phosphate as the tri-, di-, or monobasic salt was added in just sufficient concentrations to give a neutral reaction with phenolsulfonephthalein (pH = 7.7), and the results were then compared with that of the original chemical alone.

For each substance used, at least four different tests were made with human blood, five with rabbit blood, and two or three, when possible, with guinea pig and dog blood, so that the mean of the several bloods of each species might be used as the final count, providing there was little individual variation. Since the degree of leucopenia or leucocytosis made a great difference in the exact number of leucocytes adhering, though not the relative number, as a routine matter white cell counts were made on the blood of all animals every week, since it was found that in health there is little variation in that length of time. It was found that human blood varied markedly before and after eating, so certain times after eating were chosen for drawing blood when possible, usually $2\frac{1}{2}$ to 3 hours after a meal.

An interesting observation is that immediately after eating, within 30 minutes, the leucocytes stick much more readily to the

agar than at other times. This holds good for all the species tried. This has been noted for leucocytes on glass slides by several investigators. Cohnheim, among others, mentions the difference in behavior of leucocytes at different times of the day, but the reasons for this have never been shown.

The experimental animals were kept from food for about 3 hours before use. When possible the same animals were used with the different drugs. It was found that with a 2 per cent solution of salt-free agar about 4 per cent of the leucocytes would always adhere to the agar. This was constant with the blood of all the animals employed.

The following chemicals were used:

1. Calcium salts, as the chloride, carbonate, tartrate, citrate, lactate, sulfate, cyanide, and cinnamate.
2. Sodium salts, as the chloride, carbonate, tartrate, citrate, lactate, sulfate, cinnamate, and tri-, di-, and monobasic phosphates.
3. Potassium chloride, acid carbonate, and acid phosphate.
4. Barium sulfate and sulfide.
5. Strontium chloride.
6. Magnesium sulfate, tartrate, citrate, and these compounds mixed with strontium salts.
7. Mercury chloride, nitrate, subsalicylate, and iodate.
8. Organic acids, as oleic, lactic, uric, and nucleinic.
9. Amino-acids and amines, as tyrosine, histidine, glycine, alanine, glutamic acid, aminovalerianic acid, tyramine, and histamine.
10. Alkaloids, as morphine and morphine sulfate, hydrochloride, nitrate, and citrate; codeine and codeine hydrochloride; heroine and heroine hydrochloride; caffeine sulfate, quinine sulfate, strychnine sulfate, and brucine sulfate.
11. Miscellaneous substances, as papayotin, or papain, urea, urethane, creatinine, ethyl alcohol, chloral hydrate, cantharidinum, aspirin (acetyl-salicylic acid), chloretone, glucose, "mustard gas" (dichloroethyl sulfide) "parazol" (dinitrodichlorobenzene), olive oil, scarlet R (purified), turpentine, veronal (diethylmalonylurea), yeast vitamine (Harris), acetanilide, and ether.

EXPERIMENTAL.

Calcium Salts.

All calcium salts were positively chemotactic for leucocytes except calcium citrate. This was neutral in a 1 per cent molecular concen-

tration, but all other concentrations were negatively chemotactic, the citrate ion overpowering the calcium ion.

Calcium chloride showed a marked increase in the number of cells adhering to the agar with which it was combined, as compared with the control. When there was more than 2 per cent molecular concentration, there was a precipitate through which the light could not penetrate, so that the cells could not be counted when the floor was composed of agar containing the same concentration of the salt as the margins.

An illustrative series of experiments with calcium chloride is given in detail below. The figures in the top row are the percentage of calcium chloride contained in the water agar solution of each cell, and this represents the molar or gram-molecular concentration of calcium in calcium chloride. Thus in the case of a 5 per cent solution 2.75 gm. of calcium chloride would be used with 20 cc. of a 2 per cent solution of water agar, so that there would be 1 gm. of calcium present in 20 cc. of the agar solution. An exact count was made of the number of cells migrating into the agar and adhering, after the clot had been removed and the window and floor of the cell washed with normal salt solution, and this is recorded in each of the remaining rows. Each row gives results obtained with a separate sample of blood. The lowest row of figures in each group is the mean of the other figures in that column.

In the second group of figures, from observations with the floor as well as the margins of the cell formed of agar plus the different concentrations of calcium chloride, it will be seen that many more cells adhere in concentrations of 0.5 to 2 per cent than when only the margins of the cell contained calcium chloride. When frozen sections were made of these different agar plates it was found, after examination of the serial sections, that the leucocytes migrated into the agar to the depth of 0.68 to 0.84 mm. when calcium chloride was combined with the agar, while the migration into the plain agar occurred only to the depth of 0.22 to 0.47 mm. when the margins contained the calcium chloride.

Calcium Chloride Combined with Agar. Floor of 2 Per Cent Plain Agar. Human Blood.

	5 per cent.	3 per cent.	2 per cent.	5 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.08 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	Control.
	2,108	3,149	2,508	3,097	3,146	3,564	4,036	4,587	4,562	3,736	2,836	2,564	2,573	1,892
	1,872	2,569	3,095	2,764	3,593	4,095	4,539	3,842	4,594	3,874	2,893	2,482	2,531	1,936
	1,869	2,546	3,148	3,056	3,582	4,082	3,584	3,792	4,327	2,746	2,683	2,538	2,443	2,180
	2,304	2,492	3,345	3,543	4,726	5,238	5,098	5,070	4,956	4,086	3,082	2,775	2,132	2,038
	2,495	3,178	3,487	3,642	4,946	5,654	5,187	5,136	5,032	4,301	3,149	2,867	2,845	2,645
	1,657	3,482	3,542	5,035	5,692	5,237	5,109	5,192	5,017	4,785	3,376	2,850	2,742	2,543
Average	2,051	2,901	3,187	3,523	4,260	4,645	4,591	4,603	4,746	3,921	3,003	2,696	2,546	2,205

Floor of 2 Per Cent Agar Plus Calcium Chloride. Human Blood.

	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.08 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	Control.
	8,324	7,891	8,513	7,549	4,079	5,538	3,175	2,486	2,568	3,125	3,146
	5,876	5,683	5,962	5,872	3,762	4,163	2,104	1,973	2,015	2,047	1,985
	5,769	5,468	5,870	5,637	3,652	4,075	2,346	2,074	2,016	2,168	2,042
	5,678	5,469	6,085	5,364	3,869	4,197	2,834	1,985	2,175	2,068	2,164
	5,697	5,683	6,197	5,639	3,749	4,329	2,096	2,438	1,986	2,157	2,352
	6,107	5,789	5,985	6,097	3,578	4,097	2,189	2,357	2,439	2,146	2,458
Average	6,242	5,997	6,452	6,115	3,761	4,396	2,457	2,235	2,206	2,260	2,356

Calcium tartrate was slightly positively chemotactic, there being an increase of 25 per cent in the number of cells adhering at 0.25 per cent molecular concentration, as compared with the control.

Calcium lactate was slightly positively chemotactic despite the negative influence of the lactate ion, there being an increase of 30 per cent over the control at 0.1 to 0.05 per cent molecular concentration.

Calcium cyanide was slightly positively chemotactic when the floor of the cell consisted of plain water solution of agar and the sides of the window consisted of 2 per cent water solution of agar plus calcium cyanide. When both the floor and the sides of the

cell were made of the agar containing the cyanide, there was a slightly negative chemotaxis. This may possibly be explained on the basis that calcium cyanide, except in minute quantities, is highly toxic, and only in these small quantities does the chemotactic influence of the calcium overcome the cyanide poisoning. It has been because of these variations in action that the plain slab and the salt-containing slab have been used in every case. This will be referred to later. The average figures obtained in five experiments with calcium cyanide and human blood are given below:

Calcium Cyanide.

1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
3,614	3,548	4,018	3,564	3,532	3,147	2,591	1,403	2,017
—	—	2,018	2,136	547	536	241	218	2,106

In all the following tables, the first row of figures will be the average of five experiments for those cells in which the floor of the cell consisted of salt-free water solution of agar, the lower rows of figures will be for those cells in which both the floor and walls were composed of agar containing the tested reagents. Because of space restriction only a very small proportion of the actual results are published, for the purpose of illustrating the procedure.

Calcium carbonate, calcium sulfate, and calcium cinnamate were all somewhat positively chemotactic and varied very little from each other. The greatest number of leucocytes adhering was when the molecular concentration was 0.25 per cent. there being an increase of 50 per cent over the control.

Calcium citrate was negatively chemotactic in all concentrations over or below 1 per cent, as mentioned previously. A typical set of average figures for human blood with calcium citrate follows:

Calcium Citrate.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control
1,507	2,464	3,092	2,573	2,798	1,549	1,485	1,502	3,017
1,673	2,793	3,052	2,489	2,538	1,537	1,426	1,507	3,164

Sodium Salts.

The sodium salts had no constant action that depends on the sodium ion *per se*. Sodium chloride was positively chemotactic at 1, 0.8, and 0.75 per cent molecular concentrations, nearly twice as many leucocytes adhering as in the control slides with the blood of the same animal. In more than 5 per cent molecular concentration there was a negative chemotaxis, only two-thirds to one-half as many cells adhering as in the control slides. These figures applied to all blood, human, rabbit, dog, and guinea pig. A typical set of figures obtained ina series of experiments with sodium chloride is shown below:

Sodium Chloride.

7.5 per cent.	5 per cent.	4 per cent.	3 per cent	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	Control.
1,042	1,965	2,386	2,576	3,091	4,027	3,957	2,538	2,273	2,015	1,869
1,036	1,538	2,069	2,537	3,096	4,586	4,097	2,437	2,097	2,175	2,013

Sodium sulfate was neutral in regard to chemotaxis in all concentrations below 4 per cent molecular concentration for all animals. Above this concentration, it was negatively chemotactic. Sodium carbonate was neutral in almost all concentrations, although with from 0.8 to 1.2 per cent molecular concentration there was a slight positive chemotaxis. Sodium tartrate was neutral for almost all concentrations. Sodium citrate was neutral in all concentrations below 0.1 per cent; above this it was negatively chemotactic for human blood; for rabbit blood there was a slight degree of negative chemotaxis for molecular concentrations above 0.5 per cent.

Sodium Citrate.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
Human blood.							
1,579	1,468	1,452	1,792	2,247	2,296	2,158	2,375
1,538	1,396	1,425	1,658	2,153	2,153	2,096	2,436
Rabbit blood.							
2,548	2,459	2,197	2,987	3,084	3,178	3,097	2,976
2,356	2,351	2,146	2,857	2,967	3,089	3,096	2,963

Sodium lactate was negatively chemotactic in all concentrations for human blood, but with rabbit blood there was no negative chemotaxis, and with the blood of three of the five animals, there was an increase of about 25 per cent in the number of leucocytes adhering at 0.05 to 0.1 per cent molecular concentration.

Sodium cinnamate was somewhat positively chemotactic in molecular concentrations of 2 per cent and over. In lower concentrations it was neutral. This held true for the blood of man, rabbits, and dogs.

Sodium phosphate was positively chemotactic in varying degrees as the tri-, di-, and monobasic salt.

In the work on sodium phosphate, advantage was taken of the fact that pneumonia patients who had received large doses of sodium phosphate either by mouth or intravenously showed an increased number of leucocytes migrating into the agar containing sodium phosphate or calcium chloride. A normal person was used as control and the blood tested at intervals after the same doses of sodium phosphate. It was found that, alike in pneumonia and in health, many more cells migrated into agar containing Na_2HPO_4 or $CaCl_2$ than in the case of those people who had not received the sodium phosphate medication. A typical set of figures is shown below for the blood of patients who had received large doses of sodium phosphate, when tested with Na_2HPO_4 agar.

Na_2HPO_4.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
Pneumonia blood.								
6,182	7,023	7,147	10,862	7,046	7,136	7,563	7,014	5,409
11,427	15,356	15,876	18,973	14,899	14,992	7,642	7,924	5,311
Normal human blood.								
4,847	4,939	4,883	4,439	4,872	4,976	4,895	3,869	2,197
6,436	6,854	6,973	9,037	6,853	6,693	3,246	3,386	2,201

The blood of all animals showed a positive chemotaxis with Na_3PO_4 in molecular concentrations of 0.1 to 2 per cent; in concentrations

over 2 per cent there was a slight negative chemotaxis, probably due to the alkaline reaction.

With both NaH_2PO_4 and Na_2HPO_4 marked positive chemotaxis was produced, as shown by the two following tables:

NaH_2PO_4 with Normal Human Blood.

4 per cent.	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	Control.
4,387	4,097	4,028	4,538	4,538	3,327	3,107	2,145	2,047
4,038	6,359	6,109	7,537	7,634	6,094	6,024	2,214	2,046

Na_2HPO_4 with Normal Human Blood.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	Control.
10,847	5,432	7,096	7,756	4,483	3,224	2,875	2,846
9,856	8,951	9,943	8,926	6,453	4,954	4,493	2,893

When to calcium chloride NaH_2PO_4 was added, there was a marked increase in the number of cells adhering. There seemed to be a synergistic action between sodium phosphate and calcium chloride, the increase in the number of cells being greater than could be accounted for by the addition of the cells adhering with either of the two chemicals alone.

$CaCl_2$ Plus NaH_2PO_4.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
10,752	11,765	10,967	9,870	10,236	9,846	3,245	2,346
13,257	14,735	11,364	12,453	13,247	9,364	6,325	2,433

Potassium Salts.

All potassium salts were negatively chemotactic for all blood of all animals employed when concentrations over 0.01 per cent (molecular concentration) were used. This held true for potassium chloride, potassium acid carbonate, and potassium acid phosphate. In molecular concentration below 0.01 per cent there was a neutral response with all blood, human, rabbit, dog, and guinea pig.

Barium Salts.

Barium sulfide was slightly negatively chemotactic in molecular concentration over 0.05 per cent. Barium sulfate was slightly positively chemotactic.

Strontium Chloride.

Strontium chloride was slightly positively chemotactic, but strontium chloride with equal molecular concentrations of magnesium sulfate gave a great increase in the number of cells adhering to the agar. Here again the action seemed synergistic, since neither alone gave marked reaction, and many more cells adhered to the agar than could be accounted for by mere addition of the cells emerging when both salts were used.

Magnesium Salts.

Magnesium salts acted in the same way as sodium salts in regard to chemotaxis, this seeming to depend largely on the negative ion attached to the magnesium. Magnesium sulfate was neutral as regards chemotaxis in all concentrations for all blood. Magnesium tartrate was slightly negatively chemotactic in concentrations over 1 per cent, but neutral in all concentrations below this for all blood used. Magnesium citrate was slightly negatively chemotactic for all blood but more so for rabbit than for human blood.

Mercury Salts.

All mercury salts behaved about the same in regard to chemotaxis. They seemed to fix the blood before the cells could migrate into the agar, so that only a few could be counted. The clot formed was hard and brittle. This apparent negative chemotaxis of mercury salts is really a fixation of proteins and not a chemotaxis of any kind. For these reasons, the action of these compounds must be studied in some other way to determine the degree of chemotaxis they possess.

Organic Acids.

Oleic acid was positively chemotactic. This substance was used with the paraffin method so that the acid would dissolve quickly

throughout. Since, however, only the sides of the cell thus formed contained the reagent, this was unsatisfactory. For this reason, an emulsion of oleic acid with agar was made and then cooled and hardened rapidly over ice so that the floor and sides of the cell thus formed would contain the same concentration of the reagent. There was an increase of 100 per cent in the number of cells adhering at 0.5 per cent concentration over the control; in concentrations over 1 per cent there was no chemotactic effect, about the same number of cells adhering as in the control. The sodium salt of this acid showed only a very slight increase in the number of cells adhering at 0.1 per cent molecular concentration; above this there was a slightly negative chemotaxis.

Lactic acid was negatively chemotactic. This may have been due in part to the hydrogen ion concentration, but the sodium salt was also negatively chemotactic although not so markedly so as the pure acid. There were only half as many cells adhering with lactic acid in concentrations above 0.05 per cent as there were in the control.

Uric acid was slightly negatively chemotactic in molecular concentrations above 0.5 per cent; below this concentration it had no effect.

Nucleinic acid made from yeast was positively chemotactic in molecular concentrations of 0.0005 to 0.00001 per cent or percentage concentrations of 0.0065 to 0.00013; when the acidity was overcome by by the addition of a slight amount of sodium hydroxide (hydrogen ion concentration pH = 7.7), there was an increase in the number of cells adhering. With the free acid there were two-thirds more cells adhering at 0.0025 per cent molecular concentration (0.0325 per cent by weight) than in the control. With the addition of the sodium hydroxide there were twice as many cells adhering as in the control.

Amino-Acids and Amines.

Glycine and alanine were slightly positively chemotactic, alanine being somewhat more so than glycine. At 4 per cent concentration there were about three times as many cells adhering as in the control when glycine was used, while with alanine there were about three and one-half times as many cells adhering.

Tyrosine was somewhat positively chemotactic. There were about three times as many cells adhering with molecular concentrations of 0.0001 to 0.001 per cent as in the control. Tyramine, made synthetically,[6] acted most peculiarly. The cells clumped in such a way that it was impossible to count or even to estimate the number migrating into the agar with any degree of exactitude. This was repeated many times, the same thing occurring each time. No reason could be found for this action of tyramine. It suggested the appearance of a calcium cyanide clot, but differed in that the cells were much more firmly adherent to each other with tyramine than with calcium cyanide.

Histamine[7] was strongly positively chemotactic in molecular concentrations of over 0.000025 per cent (0.000055 per cent by weight), but the leucocytes of dog and human blood showed a greater degree of migration into the agar containing histamine than did the leucocytes of rabbit blood. One set of illustrative figures will be given for human, one for dog, and one for rabbit blood.

Histamine.

0.005 per cent.	0.00025 per cent.	0.0001 per cent.	0.00005 per cent.	0.000025 per cent.	0.00001 per cent.	0.000005 per cent.	0.0000025 per cent.	Control.
Human blood.								
7,021	6,538	5,028	2,537	3,159	1,138	1,274	1,485	1,407
7,183	6,983	9,894	1,093	4,963	2,915	1,019	1,857	1,396
Dog blood.								
5,379	5,027	2,473	1,532	2,036	1,463	3,527	4,037	3,016
7,037	7,239	7,635	6,528	6,017	3,012	5,172	5,187	3,423
Rabbit blood.								
4,507	4,017	3,012	1,093	1,004	1,025	1,028	857	1,046
—	4,321	3,294	993	3,653	1,946	1,956	1,845	1,243

Histidine from the same source showed a slight positive chemotaxis for all blood, human, dog, and rabbit. There was an increase of about

[6] The tyramine was made by Dr. Koessler and Dr. Hanke

[7] This was also obtained from Dr. Koessler and Dr. Hanke.

25 per cent in the number of cells adhering at 0.005 per cent over the number adhering in the control window.

Glutamic acid was slightly positively chemotactic in concentrations of 0.0005 to 0.00001 per cent; when the acidity was overcome by the addition of a slight amount of sodium hydroxide, the solution being neutral to phenolphthalein (pH = 7.7), there was an increase in the number of cells adhering. When the free acid was used there was an increase of 100 per cent in the number of cells adhering with concentrations of 0.005 to 0.00001 per cent, while with the addition of sodium hydroxide the increase was 200 per cent over the control.

Aminovalerianic acid was positively chemotactic in all concentrations to about the same extent as glutamic acid. With the addition of sodium hydroxide there was also an increase in the number of cells adhering. A typical series for aminovalerianic acid and human blood is:

Aminovalerianic Acid.

0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	0.0001 per cent.	0.00005 per cent.	0.00001 per cent.	0.000005 per cent.	Control.
3,537	4.091	4,486	3,147	2,159	2,038	1,537	2,046	3,034	1,986
6,283	5,897	5,546	4,138	5,589	3,143	3,107	3,186	3,037	2,037

Aminovalerianic acid with NaOH added to neutrality.

4,031	4,899	4,998	3,887	3,926	3,967	4,037	3,448	3,963	2,015
9,837	9,853	9,475	6,015	4,084	3,996	3,026	4,035	5,863	2,104

Alkaloids.

Morphine was slightly positively chemotactic for human and dog blood in most concentration for all morphine compounds used except the citrate. This is contrary to most of the work that has been done with morphine and its compounds, but after many repetitions the same result was obtained. There is much less increased migration of leucocytes with rabbit than with human blood. Morphine sulfate showed a slight but constant positive chemotaxis for human and dog blood in concentrations of 0.3 to 2 per cent, for rabbit blood in concentrations of 3 per cent. Morphine, morphine hydrochloride and nitrate,

all showed the same degree of chemotaxis. Morphine citrate was practically neutral in concentrations below 0.05 per cent, but above that it was negatively chemotactic.

Morphine Hydrochloride.

3 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
Human blood.										
	1,817	1,537	1,509	1,598	1,648	1,892	2,147	3,027	2,568	1,785
	2,047	5,538	5,018	5,537	5,536	5,023	4,321	4,572	4,012	1,825
Rabbit blood.										
3,018	1,016	1,546	1,512	1,478	1,443	1,976	2,314	2,215	2,415	2,648
4,001	1,192	2,032	1,472	2,351	2,511	1,897	1,998	2,322	2,451	2,771

Heroine and heroine compounds were neutral as regards chemotaxis. Codeine and codeine compounds were neutral in most concentrations, but in concentrations over 1 per cent there was a slight negative chemotaxis.

Caffeine sulfate was negatively chemotactic for all blood in all concentrations. Quinine sulfate, strychnine sulfate, and brucine sulfate were all negatively chemotactic for all blood in all concentrations.

Miscellaneous Substances.

Papayotin, or papain, was markedly negatively chemotactic for human blood in all concentrations. In fact, only 5 to 100 leucocytes adhered to the agar containing concentrations of papayotin over 0.05 per cent. With rabbit blood there was a remarkable difference, a positive chemotaxis being observed with all concentrations over 0.05 per cent. This decided chemotaxis for rabbit blood is mentioned by Gabritchevsky. Dog blood came midway between rabbit and human blood, while the blood of frogs varied with the temperature of the body; thus the winter frog blood was negatively chemotactic, the summer frog blood was positively chemotactic.

Papayotin.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	Control.
Human blood.								
120	110	100	102	135	385	732	983	2,361
16	34	35	74	52	87	128	1,184	2,752
Rabbit blood.								
6,284	6,938	6,733	5,732	3,462	3,352	2,573	2,283	4,198
6,988	6,793	6,990	5,938	4,521	3,710	3,109	2,846	4,017

Urea, urethane, and creatinine were all negatively chemotactic for the blood of all animals, in molecular concentrations above 0.5 per cent; below this concentration they were neutral.

Ethyl alcohol was negatively chemotactic in all concentrations over 2 per cent, no matter whether the paraffin or agar method was used. In concentrations under 2 per cent, alcohol was neutral.

Cantharidin, or cantharidinum (U.S.P.), was positively chemotactic. Cantharidin was obtained from Chinese cantharides and the purified cantharidin was dissolved in 70 per cent alcohol and an emulsion made with agar. This emulsion was cooled rapidly over ice, then the windows were cut as in previous cases. The blood of different animals varied markedly towards cantharides. This was strikingly shown for rabbit blood. The rabbit seems to have a congenital tolerance for cantharidin, and rabbit leucocytes were little influenced by cantharidin, which was decidedly positively chemotactic for leucocytes from other species.

Cantharidin.

0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
Human blood.									
5,987	3,863	3,993	7,946	5,124	2,136	1,872	2,044	2,115	2,109
1,928	6,937	4,837	8,123	5,923	1,927	1,764	2,942	2,012	2,213
Rabbit blood.									
6,001	4,336	4,118	2,119	2,139	2,003	1,947	3,024	3,642	3,798
1,125	2,453	1,362	2,774	3,632	3,587	1,176	3,531	3,346	3,631

It will be noticed that when the concentration of cantharidin was over 0.25 per cent, fewer cells migrated into the agar combined with cantharidin than into the plain agar with a margin of agar plus cantharidin. It is possible that strong concentrations of markedly chemotactic substances have a repelling action. This is shown more markedly in the case of rabbit blood, for here there seems to be a repelling action in all concentrations over 0.05 per cent in the cells with a cantharidin-containing floor.

Aspirin (acetyl-salicylic acid), acetanilide, and chloral hydrate were all negatively chemotactic in all concentrations for all blood. The reduction in the number of cells adhering varied from 20 to 60 per cent below the normal. Fewer cells adhered in concentrations over 0.5 per cent than below. At 4 per cent concentration, with all of these reagents, only about 40 per cent of the control number of leucocytes adhered.

Chloretone (Parke, Davis and Company) was slightly positively chemotactic in practically all concentrations, the increase varying from about 5 to 20 per cent over the control.

Glucose was neutral in all concentrations under 20 per cent; above that there was a very slight negative chemotaxis.

Mustard gas (dichloroethyl sulfide) was practically neutral in all concentrations. An emulsion was made of the sulfide in oil with agar and then cooled rapidly over ice. When the sulfide in oil was added to paraffin, more of the gas volatilized because the oil had to be added at a much higher temperature, due to the higher melting point of the paraffin than of the agar. With paraffin, also, the floor consisted of the plain glass so that the effects of the reagent could be seen only along the sides of the window thus made.

Parazol, or crude dinitrodichlorobenzene,[8] was somewhat positively chemotactic in concentrations under 1 per cent; over that it was negatively chemotactic.

Olive oil was neutral in all concentrations.

Scarlet R, purified, was slightly positively chemotactic in concentrations of 0.5 to 2 per cent, below that it was neutral.

[8] Concerning the toxicity and properties of parazol see Wells, H. G., *J. Ind. Hyg.*, 1920, ii, 247; Voegtlin, C., Livingston, A. E., and Hooper, C. W., *Bull. Hyg. Lab., U. S. P. H., No. 126*, 1920, 183.

Turpentine was slightly positively chemotactic in concentrations over 0.1 per cent; there was an increase of 50 per cent over the control at 0.5 per cent concentration.

Veronal was slightly negatively chemotactic in all concentrations over 0.5 per cent; below that it was about neutral.

Yeast vitamine (Harris) was slightly positively chemotactic in concentrations over 0.1 per cent. This may be due to the fact that many amino-acids and extractives are contained in this substance, and as they are positively chemotactic, anything in which they are found will show a certain degree of positive chemotaxis.

Ether was slightly positively chemotactic in concentrations over 0.1 per cent, when there was an increase of about 50 per cent over the control in the number of cells adhering.

DISCUSSION.

This new method of Wright's has been found very satisfactory for the determination of chemotaxis *in vitro*. It has been modified somewhat; *i.e.*, there is no need for calibrated capillary pipettes, as ordinary capillary pipettes may be used and 1 or 2 drops from the same pipette will give the same amount of blood. Cooling will cause changes in the reaction, so that it is necessary to keep the blood at 37°C. at all times. The hydrogen ion concentration is an important factor and this has been considered carefully.

This method is definitely quantitative. All steps may be carefully controlled, so that factors of error may be practically eliminated. Substances may be used with this method that have heretofore never been studied as regards their chemotactic powers; among these are cantharidin, histamine, mustard gas, parazol, and tyramine. The results obtained by this method with substances frequently studied, agree in practically all particulars with those obtained by other workers with different methods. It may also be employed as a method of determining the action of unknown drugs in producing the migration of leucocytes at the site of injection. The results obtained with sodium phosphate compounds, showing them to be strongly positively chemotactic and increasing the chemotaxis of the blood of those taking them either by mouth or intravenously, are

of interest in relation to recent work on the use of these salts in the treatment of many infections.

It has been found that the blood of different species of animals may react differently towards different drugs; thus, rabbit blood shows much less positive chemotaxis for cantharidin than human or dog blood. On the other hand, rabbit blood was much more markedly positively chemotactic for papayotin than human or dog blood. With histamine, human and dog blood showed more cells migrating into the agar than did rabbit blood. There seems to be no apparent underlying reason for this difference in action of the blood of different animals.

CONCLUSIONS.

1. Wright's method for the study of chemotaxis of leucocytes *in vitro*, slightly modified, has been found to be most satisfactory in the estimation of the degree of chemotaxis of various substances, because it is possible to make an exact quantitative determination of the leucocytes that have migrated from the blood clot and adhere to the surfaces containing the tested substance.

2. The calcium ion is the only inorganic ion *per se* which is found to be positively chemotactic under the conditions of these experiments. It is markedly chemotactic in all concentrations and in all combinations, except the citrate. Here the negative chemotaxis of the citrate ion neutralizes the positive chemotaxis of the calcium ion, and neutrality of chemotactic effect results.

3. The sodium and magnesium ions themselves are neutral. Magnesium and sodium salts are dependent upon the negative ion with which the magnesium or sodium is combined for such positive or negative chemotaxis as is exhibited. All the phosphates of sodium, whether tri-, di-, or monobasic salts, are markedly positively chemotactic, and when combined with other reagents which are themselves neutral or negatively chemotactic, produce marked positive chemotaxis. The blood of a person who has taken phosphates either by mouth or intravenously shows a great increase in chemotaxis with sodium phosphate, with calcium chloride, and even with sodium chloride which is ordinarily neutral.

4. All potassium salts are negatively chemotactic.

5. Many substances act synergistically as regards chemotaxis; *e.g.*, when strontium and magnesium salts are mixed there is a marked increase in chemotaxis. Sodium phosphate acts synergistically with calcium chloride.

6 Mercury salts fix the leucocytes in this method so that their influence on chemotaxis cannot be determined.

7. Morphine and morphine salts are positively chemotactic; this is contrary to the results obtained by others with different methods.

8. Substances which produce a very acute inflammation, such as cantharidin, histamine, or turpentine, are found to be positively chemotactic by this method, but substances, such as mustard gas, which produce a marked necrotizing effect are found to be negatively chemotactic, or neutral, though physiologically they would appear to be positively chemotactic.

9. All amino-acids and amines are positively chemotactic to a certain extent. It seems that the longer the carbon chain, the greater the degree of chemotaxis, though this is not absolute. Tyramine is one exception to this, for it causes a peculiar clumping of the cells, so that it is impossible to count the number adhering, and thus determine whether or not tyramine is positively chemotactic.

10. The time that the blood of animals is examined after eating makes a marked difference in the number of cells adhering, for shortly after eating, within 30 minutes, very many more cells will adhere to the agar than at a later time.

11. The blood of different species of animals reacts differently towards different reagents. The chemical composition of these agents seems to have nothing to do with this difference in reaction as far as we could determine.

12. With frozen serial sections it has been found that the depth of penetration of the leucocytes into the agar is proportional to the positive chemotaxis produced by the substance combined with the agar, as demonstrated by the number of leucocytes adherent to the walls of the test chambers.

OBSERVATIONS ON THE RELATION OF UREA TO UREMIA *

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The objects of this research were to determine what relationship existed between the toxicity of various normal urinary constituents and the manifestations of what is clinically known as uremia—meaning by the term "uremia" the symptoms observed as a result of marked obstruction to the outflow of urine, on the one hand, and, on the other hand, the symptoms which appear in many cases of chronic Bright's disease, characterized by convulsions, various paralyses and coma.

In pursuance of these objects, it was thought advisable to begin with the simplest organic urinary constituent—urea—to determine its toxicity on dogs—when injected in large doses in relatively short periods of time—to note the symptoms produced, to follow the changes in the blood and urine content of urea, and to determine, at the necropsy, the pathologic changes that might have taken place—both gross and microscopic—as well as the urea content of various body fluids. Then, the toxicity of urea was determined in dogs in which one ureter had been ligated; and in other cases both ureters were ligated and the changes in the blood urea followed until the death of the animal.

THE LITERATURE

There is a tremendous literature [1] on the subject of uremia and related topics; but, while theories are abundant, and there are many facts, there is a great deal of conflict of opinion on every aspect of the subject—even on the subject as to what constitutes "uremia."

One of the earliest theories of the causation of uremia was a chemical theory put forth by Wilson in 1833, in which the symptoms were ascribed to the retention of urea in the blood. But discrepancies were soon noted between the amounts of urea in the blood and the severity of the symptoms in patients, and later workers, Frerichs, in 1852, Feltz and Ritter, in 1880, Bouchard, in 1887, Herter, in 1898, and many others, found that urea was relatively nontoxic, when injected into animals. In fact, Bouchard, using rabbits, found that the amount of a concentrated urea solution necessary to produce severe symptoms was equal to the toxic dose of distilled water itself. Although Gallois, Richardson, in 1862, Voit, in 1868, and others, claimed positive results,

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1. For most of the literature up to 1900 we have made use of Ascoli's "Urämie" (1902) in which a complete review of the subject is given.

they were met with the objection that their results were due, not to the urea itself, but to various impurities or to the osmotic changes induced by the concentrated solutions employed.

An anatomic theory, suggested by Traube in 1860, ascribed uremia to edema of the brain and consequent circulatory disturbances. But necropsies soon disclosed the fact that great inconsistencies existed between the anatomic and clinical findings, so the theory lost ground, and a return to a chemical theory followed. This time ammonium carbonate was blamed, Frerichs, in 1851, postulating that urea in the blood was converted by ferments into the toxic ammonium compound, while Treitz, in 1859, believed that the change occurred in the intestines. However, the ammonia content of uremic blood was found to be negligible.

Voit, in 1868, Feltz and Ritter, in 1881, and Linbeck, in 1898, turned their attention to the inorganic constituents of urine, and chose potassium chlorid, because of its extreme toxicity to animals, as the cause of the symptoms of uremia—especially the convulsions and coma. However, the clear-cut cardiac picture in potassium poisoning, and the inability to demonstrate a constant increase in the potassium content of the uremic blood, spoke against this theory. Schottin, in 1853, found an increased amount of extractives in the blood of uremics—especially creatin and creatinin. Jaccoud, in 1867, had a similar experience, and Landois, in 1891, had worked up the subject very thoroughly and produced convulsions in rats, dogs and apes by the direct application of creatin to the cerebral cortex. Feltz and Ritter, however, had to inject 2 gm. creatinin intravenously before they could produce convulsions and death, and these they obtained only in one dog. Various salts of creatin and creatinin, in from 5 to 6 gm. doses, had no effects. Various other extractives—uric acid, hippuric acid, leucin, tyrosin, purin bases, phosphates, sulphates and chlorids—were similarly ruled out as a possible cause of uremia.

Still clinging to the idea of urinary retention as the cause of uremia, Bouchard, from 1882 to 1889, worked on the toxicity of whole urine when injected into rabbits. He found human urine quite toxic, and could account for only half of this toxicity on the basis of potassium salts. He could extract various substances from human urine, which, on injection into rabbits, produced any or all of the symptoms of uremia. The urine of nephritic patients was found to be much less toxic than normal urine—due, presumably, to retention of the toxic products. Bouchard calculated that in twenty-four hours an average man excreted sufficient toxins in his urine to kill him in fifty-two hours, were all these toxins not eliminated. But Fowler, in 1881, reported many cases of complete mechanical obstruction to the outflow of urine, the patients living for many days and even two weeks without any other symptoms than asthenia and drowsiness; hence,

there was a great difference between uremia and anuria. Bouchard's work, on the whole, was very crude, and it was to be expected that he could obtain almost any symptoms by injecting such a complex of unknowns and unknowables as is present in human urine.

Brown-Sequard, in 1889, published his theory on internal secretions and included the kidney as one of the organs of internal secretion, ascribing uremia to failure or lack of such a secretion. In 1893, he stated that while nephrectomized animals lived only thirty hours on the average, similar animals to whom kidney extracts were given lived sixty hours. Meyer, in 1893 and 1894, reported that renal extracts had a marked effect on the pulse and respiration of anesthetized animals that were nephrectomized, changing the respiration from Cheyne-Stokes to normal. These results were obviously trivial and unimportant, and the theory of internal secretion of the kidney was dropped for a time.

Ascoli could see no very close relationship between the nonprotein nitrogen of the blood and the severity of symptoms, although some parallelism might be made out. Perhaps, both are merely the results of nephritis and, hence, a causal relationship between the two need not exist. In 1902, Ascoli described nephrolysins—substances having specific toxic effects on the kidneys, with side effects on nervous tissue, substances akin to immune bodies and produced by using renal tissue as antigen—and even isonephrolysins, produced when a kidney is injured for some reason or other,—and explained all the symptoms of what he called "true uremia without retention"—in contrast to "urinary intoxication with retention"—on the basis of a general intoxication with nephrolysins. He believed that nephrolysins could cause the increased blood pressure, the localized palsies, the edema, the pericarditis, stomatitis and colitis, the convulsions, the coma—in short, the classical picture of a clinical uremic attack. Ascoli emphasized especially the difference between the uremia resulting from mechanical obstruction of the urinary tract and the uremia found as a terminal event in cases of chronic nephritis—something which previous investigators had not quite clearly understood, and which was the cause of much unnecessary dispute and inconsistent experimental work. Every author had a different idea of uremia, and it is no wonder that no two authors agreed in their results.

With the advent of physicochemical investigations, many men turned their attention to the relationship between physicochemical changes in the blood and uremia. Honigmann [2] reviewed the literature in 1902. Koranyi found that the molecular concentration of the blood increased in uremia—using cryoscopy. Lindemann found a definite increase in the depression of the freezing point of water (Δ) of the blood of

2. Honigmann: Ergeb. d. Pathol. **8**:549, 1902.

uremic patients and dogs with both ureters ligated, and believed that the change in osmotic pressure was the chief cause of trouble. In an exhaustive monograph, Strauss, in 1902, found an increased Δ of the blood—usually in chronic interstitial nephritis—and a retention of organic molecules till the limit of the patient's tolerance to poisonous metabolites was reached, when any one or several of many factors might produce the uremic intoxication. Bickel, in 1902, found no change in the electrical conductivity of the blood of nephrectomized animals, and, with an increased Δ, he concluded that there must be a retention of organic molecules. However, in all these cases, no constant relationship could be found between the changes in Δ and other physical constants and the severity of the symptoms, so that, as Honigmann correctly concluded, no single series of facts—chemical, physical, or otherwise—had yet stood in constant parallelism with the clinical picture of uremia. The new findings were simply concomitant symptoms, so to speak, and not etiologic factors. Yet Stern [3] was willing to ascribe uremia to diminished electrical conductivity of the blood serum.

Bradford, in 1899, excised varying amounts of renal tissue in dogs, and found an increased output of nitrogen in the urine—due, he thought, to increased nitrogenous catabolism in the muscles. Others misinterpreted his views as favoring an internal secretion for the kidney. Pearce,[4] in 1908, removed one half, two thirds and three fourths of the renal tissue in dogs, but found no change in nitrogenous metabolism. But removal of more than three fourths of the total renal tissue led to metabolic upset, owing to starvation and gastro-intestinal disturbances. This work was well controlled, and the further work of Pearce [5] and Pearce and Sawyer [6] helped to disprove completely two theories—the internal secretion theory and Ascoli's nephrolysin hypothesis. Pilcher, in 1913,[7] extirpated renal tissue by ligation of the renal vessels—instead of excision of kidney tissue—and obtained results entirely confirmatory of Pearce's work.

When theories begin to fail, people turn their attention to facts. In scientific work much more progress may be made by the invention of new methods than by modifications of old ones, and so, in the work on uremia and chronic nephritis, the development of, perhaps, the most interesting phase of the problem—the retention or accumulation of waste products, especially nitrogenous, in the blood—was made possible by the new, delicate methods of blood and urine analysis

3. Stern: Med. Rec. **63**:121, 1903.
4. Pearce: J. Exper. M. **10**:632, 1908.
5. Pearce: Arch. Int. Med. **2**:77 (July) 1908.
6. Pearce and Sawyer: J. M. Res. **19**:269, 1908.
7. Pilcher: J. Biol. Chem. **14**:389, 1913.

introduced by Folin and his collaborators[8] since 1912, and extended by Marshall,[9] Van Slyke and Cullen,[10] Myers and Fine,[11] and many others. As a result, much of the older work has been discounted, and advantage is taken of the new methods by clinicians and laboratory men alike, in order to help accumulate sufficient data from which to draw definite conclusions.

It was suspected very early in the nineteenth century that a retention of nitrogenous waste products occurred in the blood in nephritis. The methods of investigation, however, were very crude, and the results inaccurate. Among those who worked on the nitrogen content of the blood of uremic patients, ordinary nephritics and experimental animals, were Wilson, Bostock, Frerichs, Treitz, Ascoli, Van Noorden, Schotten, Oppler, Hoppe-Seyler, Jaccoud, Landois, and a host of others.[12] Strauss, in his monograph, gave the nonprotein nitrogen of normal blood as from 20 to 35 mg. per 100 c.c.; in chronic parenchymatous nephritis, 40 mg. or over; in chronic interstitial nephritis, 85 mg. and over, reaching higher values, in uremia. Hohlweg, in 1911,[13] found 41 to 60 mg. of "residue" nitrogen per 100 c.c. in normal sera, of which 60 per cent. was urea nitrogen, as high as 120 mg. in nephritis, and up to 340 mg. in uremia. Other conditions showed no such changes. Foster, in 1912,[14] found from 43 to 164 mg. nonprotein nitrogen in chronic nephritis; from 53 to 393 mg. in uremia, with the urea nitrogen varying from 36 to 90 per cent. of the nonprotein nitrogen. Nonprotein nitrogen of 100 mg. (per 100 c.c.) or more was considered a poor prognostic omen.

With newer methods, the following results were obtained: Folin and Denis, in 1913,[15] found, in normal people, nonprotein nitrogen from 22 to 26 mg.; urea nitrogen, from 11 to 13 mg.; in chronic nephritis, nonprotein nitrogen of from 40 to 96 mg.; urea nitrogen of from 19 to 68 mg. Farr and Austin, in 1913,[16] found, in nonnephritic cases nonprotein nitrogen from 15 to 43 mg. with urea nitrogen of from 50 to 60 per cent. of the former: no change in uncomplicated cardiovascular cases, some increase in chronic parenchymatous

8. Folin and Farmer: J. Biol. Chem. **11**:493, 1912. Folin: Ibid. **11**:507, 1912. Folin and Macallum: Ibid. **11**:523, 1912. Folin and Denis: Ibid. **11**: 527, 1912.

9. Marshall: J. Biol. Chem. **14**:283, 1913; ibid. **15**:487, 1913; ibid. **15**: 495, 1913.

10. Van Slyke and Cullen: J. Biol. Chem. **19**:211, 1914; ibid. **24**:117, 1916.

11. Myers and Fine: J. Biol. Chem. **20**:391, 1915.

12. Obermayer and Popper: Ztschr. f. klin. Med. **72**:332, 1911.

13. Hohlweg: Deutsch. Arch. f. klin. Med. **104**:216, 1911.

14. Foster: Arch. Int. Med. **10**:414 (Sept.) 1912.

15. Folin and Denis: J. Biol. Chem. **14**:29, 1913.

16. Farr and Austin: J. Exper. M. **18**:228, 1913.

nephritis; nonprotein nitrogen from 40 to 180 mg. in chronic nephritis with hypertension. The nonprotein nitrogen was usually, but not constantly, high in uremia. Tileston and Comfort, in 1914,[17] studied 142 cases of nephritis and many other conditions—the latter as controls—and considered as the normal nonprotein nitrogen 30 mg. or less, with 50 per cent. urea nitrogen. In uremia, the nonprotein nitrogen was as high as 324 mg., urea nitrogen, 70 per cent. or more. Nonprotein nitrogen over 100 mg. was practically never found by them in conditions other than uremia; when occurring it was a very serious sign, and was apt to be followed very soon by death. Patients with a high nonprotein nitrogen were poor surgical risks.

McLean and Selling, in 1914,[18] gave the normal nonprotein nitrogen as from 23 to 36 mg. per 100 c.c., the urea nitrogen as from 10 to 23 mg. Foster, 1915,[19] found an average nonprotein nitrogen of 63 mg. in parenchymatous nephritis, with 84 mg. in the chronic interstitial variety, and 135 mg. in "convulsive uremia." The uric acid—normally from 3 to 5 mg. per 100 c.c.—averaged 12 mg. in uremic patients, but there was no concordance between the rise in purine and nonprotein nitrogen. In four convulsive cases, the creatin and creatinin ran from 27 to 46 mg. per 100 c.c., indicating a rather marked retention. Cullen and Ellis, 1915,[20] compared the urea nitrogen in blood serum and spinal fluid respectively, and found in the former a range of from 20 to 42 mg., in the latter from 22 to 46 mg., a very close correspondence, it is seen, and indicating the marked diffusibility of urea.

Schwartz and McGill, 1916,[21] studying the blood urea, gave, as the normal for human blood, 25 mg. urea per 100 c.c.; in acute nephritis, twenty-five cases, an average of 57.8 mg. was obtained; in mild cases of chronic nephritis, 28.8 mg. with 44.4 mg. in advanced cases, and 52.3 mg. in advanced cases with complicating cardiac decompensation. The urea content of blood and other body fluids was found to be very similar. Kast and Wardell,[22] 1918, in a study of 244 hospital cases with a urea nitrogen of less than 35 mg., concluded that 20 mg. was the upper normal limit for blood urea nitrogen, thus agreeing pretty closely with most of the previous figures. Finally, Gettler and St. George,[23] 1918, in a review of 600 cases of primary nephritis, found the nonprotein nitrogen to range between 40 and 460 mg. as compared with the normal of from 25 to 40 mg., while the

17. Tileston and Comfort: Arch. Int. Med. **14**:620 (Nov.) 1914.
18. McLean and Selling: J. Biol. Chem. **19**:31, 1914.
19. Foster: Arch. Int. Med. **15**:356 (Sept.) 1915.
20. Cullen and Ellis: J. Biol. Chem. **20**:511, 1915.
21. Schwartz and McGill: Arch. Int. Med. **17**:42 (Jan.) 1916.
22. Kast and Wardell: Arch. Int. Med. **22**:581 (Oct.) 1918.
23. Gettler and St. George: J. A. M. A. **71**:2033 (Dec. 21) 1918.

urea nitrogen, normally from 10 to 18 mg., ranged from 20 to 375 mg. In 120 cases of gout, the nonprotein nitrogen was from 38 to 55 mg. and urea nitrogen from 15 to 35 mg.—somewhat increased. They stated that where the nonprotein nitrogen was 80 mg. or above, increased protein catobolism, or failing, or poor, circulation could not be blamed as the cause, but the only possible cause was some renal trouble.

An interesting fact was pointed out by Chace and Myers,[24] 1916, who showed that in the various stages of a clinical nephritis, uric acid was retained first, then urea and, last of all, creatinin, so that one might have uric acid from 6.1 to 9.5 mg. with normal urea and creatinin, giving a "steplike" picture. In fatal cases, the uric acid ranged from 8.7 to 22.4 mg., urea nitrogen from 144 to 263 mg., and creatinin from 11.0 to 22.2 mg. All patients with a blood creatinin of more than 5 mg. died in a short time. They record thirty-four cases in which the creatinin ranged from 4.9 to 33.3 mg. Myers and Killian,[25] 1916, reported ninety-four cases with creatinin values of more than 5 mg., of which number eighty-three patients had died in a short time—80 per cent. in less than two months. They believed that the blood creatinin determination furnished a most reliable prognostic test in nephritis or uremia.

From the experimental side, it is interesting to note that in uranium nephritis—in cats—a marked retention of nonprotein nitrogen and urea nitrogen occurs; so also in cantharidin nephritis and less so in chromate nephritis, as reported by Folin, Karsner and Denis,[26] in 1912. Later on, in 1914, Karsner and Denis [27] produced a marked retention in acute nephritis as the result of injections of specific hemolytic immune serum, diphtheria toxin, and, especially, tartaric acid—nonprotein nitrogen 382 mg. in one cat. Tubular changes seemed a little more important in producing retention than glomerular changes, but definite conclusions could not be drawn. Removal of from one half to two thirds of the total kidney substance resulted in only a slight nitrogen retention for from twenty-four to seventy-two hours, but complete nephrectomy was followed by a progressive increase in nonprotein nitrogen of the blood—the values ranging from 227 to 285 mg. in three dogs reported by Karsner and others,[28] 1915.

Another evidence of retention in chronic nephritis and uremia is afforded by the study of the blood indican. Obermayer and Popper,[12] in 1911, believed that the presence of indican in the blood was specific

24. Chace and Myers: J. A. M. A. **67**:929 (Sept. 23) 1916.
25. Myers and Killian: Proc. Soc. Exper. Biol. & Med. **16**:41, 1918.
26. Folin, Karsner and Davis: J. Exper. M. **16**:789, 1912.
27. Karsner and Denis: J. Exper. M. **19**:259, 1914.
28. Karsner, Bunker and Grabfield: J. Exper. M. **22**:544, 1915.

for uremia. In thirty-eight uremic patients they found a blood indican of from 3.7 to 6.0 mg. per 100 c.c. They did not ascribe any etiologic rôle to indicanemia, but considered it a delicate indicator of retention. Tschertkoff,[29] in 1914, found indicanemia in many cases of nephritis without uremia. Haas,[29] in 1914, using better methods, determined 0.045 mg. per 100 c.c. as the amount of indican in the blood of normal individuals, from 0.06 to 0.145 mg. occurring in gastro-intestinal affections, from 0.2 to 2.7 mg. in nephritis with definite "renal insufficiency." Indican was increased long before the nonprotein nitrogen and was a better indicator of impending "renal insufficiency" than either the nonprotein nitrogen or urea nitrogen—a value of 0.180 mg. being a very suggestive sign of impending trouble. However, injection of large doses of indican into dogs and man—up to 40 mg.—proved perfectly harmless, as might be expected.

It was early discovered that in the late stages of renal diseases there occurs a state of acidosis, or decreased alkali reserve of the blood. Jaksch,[2] in 1900, found a decreased alkalinity of the blood in uremia. Orlowsky,[2] 1902, found a similar change in dogs with both ureters ligated. This was considered as simply another evidence of retention. Straub and Schlayer[30] believed acidosis to be an important factor in the production of uremia, for they obtained an alveolar carbon dioxid tension of less than 35 mm. Hg., in eight cases of uremia. No acetone bodies were found. Peabody,[31] in 1915, found an acidosis—determined by the bicarbonate tolerance method of Sellards— in advanced cases of chronic nephritis; in very advanced cases the alveolar carbon dioxid partial tension fell. This acidosis was secondary to retention, and very constant in uremia. In only a few cases, however, did it cause a definite air-hunger or other symptoms. Sellards[32] had previously obtained very similar findings. Somewhat at variance with other investigators, Fischer,[33] in 1912, went as far as to call acidosis the cause of nephritis. In 1916, Marriott and Howland,[34] following the finding by Greenwald of increased total phosphorus in the serum of nephritics, reported fourteen cases of chronic nephritis and thirty-five normal people on whom blood studies had been made regarding the nonprotein nitrogen, urea nitrogen, inorganic phosphorus, calcium and magnesium. In normal people, the total inorganic phosphorus was usually less than 2 mg. per 100 c.c.; in

29. Haas: Deutsch. Arch. f. klin. Med. **119**:117, 1916.

30. Straub and Schlayer: München. med. Wchnschr. **59**:569, 1912.

31. Peabody: Arch. Int. Med. **16**:955 (Dec.) 1915.

32. Sellards: Bull. Johns Hopkins Hosp. **23**:289, 1912; ibid. **25**:141, 1914; Principles of Acidosis, 1917.

33. Fischer: Edema and Nephritis, 1912.

34. Marriott and Howland: Arch. Int. Med. **18**:708 (Dec.) 1916.

chronic nephritis without acidosis, it was from 2.7 to 5.5 mg.; in chronic nephritis with acidosis, it ranged from 8 to 23 mg. They concluded that acidosis in nephritis was due to retention of acid phosphates —a condition not found in diabetic acidosis. The rather low calcium content found might be related to the convulsions and hemorrhages of uremia; the increased phosphorus probably caused an increased excretion of calcium phosphate into the intestine.

As regards other changes in the blood, than those described above, Gabbi [2] described an increased serum globulin in rabbits and dogs with both ureters ligated. Recently, in 1916, Butterfield and others [35] studied the blood of normal people and those with cardiac and renal decompensation and edema, uremia and hypertension, for the Δ, refractive index, specific gravity, dry residue and protein content. In chronic nephritis with uremia, Δ was from 0.61 to 0.76 degrees—quite high as compared with the normal Δ—0.57; the dry residue was increased, and especially the total protein content, i. e., the serum was concentrated. When edema was also present, all the findings but Δ were lowered, due to dilution. Cases of cardiac decompensation with edema showed normal values. Rowe,[36] in 1917 found, in chronic nephritis with edema, the lowest values for total serum proteins of those found in any disease; however, the globulin was increased. Bienenstock and Csaki,[37] in 1917, made an extensive series of observations on the physicochemical changes in the blood of dogs in whom both ureters were ligated or both kidneys removed. Δ was increased in all cases, up to 0.771 degrees; the electrical conductivity of the serum was decreased; the organic molecules were increased, the serum ash was increased; the fluidity of the blood varied, as also the viscosity, surface tension and total solids. The carbon dioxid in the serum was decreased, but markedly increased in the red blood corpuscles. The H-ion concentration of the serum rose—due to undetermined organic acids.

Two suggestive studies have been made on the presence of some very toxic substance in the blood or urine. Foster,[38] in 1915, isolated a toxic base from the blood of seven uremic patients—not found in twelve controls—which caused death in guinea-pigs, preceded by dyspnea, twitchings, convulsions, coma and subnormal temperature. At the necropsy, there were found hyperemia of the brain and kidneys. Hartman,[39] in 1915, isolated an organic compound, C_6H_8O, which he called "urinod," obtainable from urine, from 1 to 2 parts in 100,000.

35. Butterfield et al.: Am. J. M. Sc. **151**:63, 1916.
36. Rowe: Arch. Int. Med. **19**:354 (Feb.) 1917.
37. Bienenstock and Csaki: Biochem. Ztschr. **84**:210, 1917.
38. Foster: Tr. Assn. Am. Phys. **30**:305, 1915.
39. Hartman: Arch. Int. Med. **16**:98 (July) 1915.

Urinod was very toxic, producing symptoms very much like those of uremia, and having the typical "urinous" odor of a uremic patient's breath. The symptoms reproduced in man and animals were chiefly of the nervous type.

The clinical classification of uremia has varied from time to time as new facts were established and old ones confirmed or disproved. Thus, Widal,[40] in 1912, described two chief types of uremic syndromes—the "chloruremic" variety, associated with retention of sodium chlorid and with symptoms of edema, diarrhea, vomiting, coma, Cheyne-Stokes breathing, delirium, convulsions and amblyopia; and the "azotemic" type in which nonprotein nitrogen retention was marked, with gastrointestinal disturbances—anorexia, vomiting, ulcerative stomatitis and colitis, pericarditis, albuminuric retinitis, stupor and torpor—with mixed pictures resulting from the predominance of urinary, vascular or cardiac syndromes.

Reiss,[41] in 1914, in an excellent review of the subject, attempted to classify uremia on a symptomatic basis, and gave three main types—the "asthenic uremia," in which predominating symptoms were drowsiness, fatigue, apathy, indifference, physical weakness, marked nonprotein nitrogen retention, and death, usually from rather sudden cardiac failure; the "convulsive or epileptiform" type, in which convulsions, unconsciousness, a high blood pressure, with little or no disturbances in the urinary secretion and practically no nitrogenous retention were characteristic symptoms; the "psychotic uremia," in which psychic symptoms, such as dementia, hallucinations, delusional insanity, deep coma and muscle twitchings, occurred, with nonprotein nitrogen not necessarily increased, but with evidence of cerebral arteriosclerosis in the majority of cases; and, finally, a mixed group in which asthenic and convulsive features were combined—by far the largest clinical group. In the asthenic variety alone were nonprotein nitrogen and sodium chlorid retention marked and characteristic. Reiss believed that the nonprotein nitrogen retention was the cause of asthenic uremia. In the other types, it could be stated definitely that the toxic substances did not arise from disturbances in the urinary tract, but were metabolic in origin.

Strauss,[42] in 1915, reserved the term "true uremia" for cases with definite retention—nonprotein nitrogen, 150 mg. or more. All the other forms, including the convulsive and arteriosclerotic types, he grouped under "pseudo-uremia"—with nonprotein nitrogen below 70 mg. Most authors now agree in recognizing at least two types of uremia—the

40. Widal: Presse méd. **20**:973, 1912.
41. Reiss: Ztschr. f. klin. Med. **80**:97, 1914.
42. Strauss: Berl. klin. Wchnschr. **52**:368, 1915.

asthenic and the toxic. Heim and Tchertkoff,[43] in 1918, described three types—one due to chlorid retention, a "true uremia" due to urea retention, and "pseudo-uremia"—the latter associated with general arteriosclerosis, and no retention, usually found after 40 years of age.

The finding of increased nonprotein nitrogen in the blood of uremic patients, and the fact that from 30 to 90 per cent. of this nitrogen was urea nitrogen, naturally led to the thought that urea was toxic. Experimentally, it was shown that double nephrectomy or ligation of the ureters produced a rise in blood urea. Thus, Herter and Wakeman,[44] in 1899, removed both kidneys in the dog, with resulting subnormal temperature, anorexia, drowsiness, vomiting and diarrhea, and death in from twenty-two to eighty-two hours. Ligation of both ureters gave similar results. In twenty-eight normal dogs, the blood urea was 37 mg. (average); in eighteen nephrectomized animals, it was as high as 315 mg. (average), and 307 mg. in animals with both ureters ligated. There were also an increase in the total proteins of the blood, and in the total phosphorus. The tissues, in general, contained from four to five times the normal percentage of nitrogen—probably urea. It might be said that earlier work on the subject is open to criticism because of the great inaccuracy of the analytical methods in vogue.

In 1914, some definite work on the toxicity of urea was done by Marshall and Davis,[45] using accurate but simple methods. Vauquelin and Segales, in 1822, were the first to record intravenous injections of urea in animals. They used small amounts and obtained negative results. Hammond, in 1858, interfered with the kidneys while injecting, and found the injections fatal. Grehant and Quinquard, in 1884, determined 600 mg. per 100 c.c. blood as the lethal dose for dogs. Herter and Wakeman, in 1899, caused the death of normal dogs by using concentrated urea solutions; urea to the extent of 1 per cent. of the body weight had to be injected to produce fatal effects. Marshall and Davis studied the distribution of injected urea, in the blood, urine, bile, spinal fluid and tissues. In normal dogs, the blood urea was from 21 to 29 mg. per 100 c.c., while the urea content of other body fluids and tissues was very similar to these figures, except in the case of fat, which was poor in urea, and the urinary tract, which was obviously rich in urea.

By injecting increasing doses of urea, in concentrated solutions, into dogs on successive days they produced, in the order named, loss of appetite, vomiting and restlessness, convulsions, coma and dyspnea, and death—the latter occurring when the amount injected reached 1 per cent. of the body weight, e. g., 25 gm. in a 2.7 kg. dog. The urea

43. Heim and Tchertkoff: Rev. méd. Suisse Rom. **38**:15, 1918.

44. Herter and Wakeman: J. Exper. M. **4**:117, 1899.

45. Marshall and Davis: J. Biol. Chem. **18**:53, 1914.

injected was excreted rapidly, and what remained was fairly evenly distributed in the blood and tissues of the body—as one would expect from the ready diffusibility of urea. There was no evidence that any of the urea was changed into other compounds.

In some animals one ureter was ligated, and it was found that, after a short time, the remaining functioning kidney could take care of as much urea as both kidneys previous to the operation, indicating a high margin of safety.

It was shown, both by injecting single large doses and repeated small doses of urea, that the tissues took up about 90 per cent. of the injected urea in a few minutes. Tremors and opisthotonic convulsions were produced when large doses were injected. At the necropsy, the brain was found to be wet and hyperemic.

Marshall and Davis concluded that the concentration of urea in the blood did not affect the ability of the tissues to absorb urea; that the kidneys had an enormous power of excreting urea—up to 40 gm. per liter; that dehydration retarded elimination, and that, in man, urea accumulated only slowly in the blood, owing to absorption by the tissues, so that it would take from four to six days of complete retention to raise the blood urea to 200 mg. Analysis of human tissues, from cases of nephritis, showed a high urea content, closely analogous to that found in the dogs. Urea was toxic only in large doses—1 per cent. of body weight—but produced symptoms in smaller amounts. It was not transformed into other compounds.

As regards the toxicity of urea in man, when urea is ingested, Hewlett and others,[46] in 1916, carried out some interesting experiments. One hundred gm. of urea, in aqueous solution, were swallowed in fifteen minutes, or in divided doses, over a period of from four to six hours. Symptoms of headache, dizziness, apathy, drowsiness, fatigue and inability to work appeared, and were most marked when the blood urea was at its maximum—from 150 to 160 mg. per 100 c.c. There were practically no symptoms between 40 and 150 mg., and symptoms always appeared after 150 mg., persisting till the blood urea had again fallen below that level. Hence, the sudden entrance of urea into the blood was not the cause. The authors noted the analogy between the symptoms produced and those seen in asthenic uremia, or in the later stages of nephritis when retention occurred.

Following a thorough review of the literature on uremia, Wells,[47] in 1920, was inclined to the view that uremia was caused by the retention of the known nitrogenous substances in the blood, emphasizing especially the importance of the time element, for the presence in the blood of a high concentration of organic substances, continuously main-

46. Hewlett, Gilbert and Wickett: Arch. Int. Med. **18**:637 (Nov.) 1916.
47. Wells: Chemical Pathology, 1920.

tained, was bound to have deleterious effects, especially when one considered the marked symptoms following even a very temporary increase in nonprotein nitrogen, if 200 mg. or more. All the pathologic findings pointed to a systemic intoxication, and varying clinical pictures might very readily be produced by variations in the retention of the several known nitrogenous constituents. Experiments with the Woodyatt machine to determine the effects of prolonged intravenous injections of the various purified urinary constituents were recommended as being highly essential for further progress.

THE METHODS OF EXPERIMENTATION

Dogs were used. They were not restricted as to food or drink. Injections of urea were made intravenously, using a buret, rubber tube and hypodermic needle, usually without cutting down on the vein—the external saphenous or some superficial thigh vein—unless absolutely necessary, and then under local anesthesia. A 33⅓ per cent. solution of urea was always used, at body temperature. No general anesthetic was necessary since the dogs lay quietly on the table, except when convulsions occurred. Blood was drawn from a leg vein, by means of a hypodermic needle attached by short rubber tubing directly to an Ostwald pipet, so that suction could be applied. In the first few dogs, the urine was obtained by means of a catheter, but later on, owing to the difficulty of inserting a catheter without assistance, and because it was not possible always to obtain female dogs, the catheter was abandoned, and samples of urine obtained only during urination and at necropsy. No attempt was made to collect twenty-four hour samples of urine.

The injections were usually continuous, except for the time it took to fill a 50 c.c. buret, and were continued until the twitchings or convulsive movements of the animal made injection difficult. Symptoms were noted carefully, at varying intervals. Necropsy was performed on all the dogs within a few hours after death, and samples of heart's blood, urine, bile, gastric or intestinal contents, taken for analysis. The viscera and the brain and spinal cord were examined in a routine manner and hematoxylin and eosin celloidin sections, as well as frozen sections for fat stains, were made.

In cases in which operative procedures were necessary—in ligating the ureters—strict aseptic precautions were observed and the anesthetic, ether, was administered by an assistant. A midline abdominal incision was employed, except in one case.

The urea in the blood was determined by mixing the drawn blood with 1 c.c. 5 per cent. sodium citrate and diluting, or not, with distilled water, if necessary, so as to give from 1 to 3 mg. of urea in 5 c.c. of the mixture or whole blood. In a large test tube, 5 c.c. of whole or diluted blood were placed, 5 c.c. of water added, 1 c.c. of 10 per cent. urease solution (freshly prepared from "Arlco" urease) and a few c.c. of paraffin oil (later a few drops of caprylic alcohol were substituted), and allowed to stand at room temperature for at least 30 minutes. This tube was connected with another tube containing from 15 to 25 c.c. of hundredth normal hydrochloric acid, some 1 per cent. solution of sodium alizarin sulphonate, and paraffin oil as above—the set of two tubes constituting part of a complete aeration apparatus, with a water suction pump to create a negative pressure. Usually, several sets of tubes were run in series. After standing thirty minutes, suction for a minute or two was carried out to get any free ammonia vapor into the acid tube; then 10 c.c. of a 50 per cent. suspension of anhydrous, finely-powdered sodium carbonate were introduced into the tube containing the fluid to be analyzed, and suction carried out for at least thirty minutes, at first a slow current for from two to three minutes and then a gradually increasing current until the maximum

aeration was obtained. Titration of the acid tube with hundredth normal sodium hydroxid and sodium alizarin sulphonate, as indicator, was done, and the urea readily estimated.

For urine, the ammonia content was first determined by simply adding 10 c.c. of a 15 per cent. potassium carbonate and 15 per cent. potassium oxalate mixture to 1 c.c. of urine, and aerating. The ammonia nitrogen was subtracted from that found in the estimation of urea. Urine was usually diluted 1:100, and so also bile and other body fluids, before determining the urea content.

Experiments with known solutions of urea were carried out until accurate results were obtained with the urease and titration method. Controls were, of course, run on the blood and other fluids for ammonia nitrogen not derived from urea, and all the reagents were carefully controlled, so that the figures given below represent the completely corrected figures. Urea was usually injected at the rate of 2 gm. per minute.

RESULTS

Owing to lack of space, the protocols of only a few of the experiments in each series are given in detail, while the remainder are abbreviated so as to include only the essential features of each experiment and any points of difference in the results of the various experiments carried out.

April 11, 1920.—Dog 1, brown female, 9.5 kg., received 30 gm. urea intravenously; vomited a few times. Blood urea rose from 20.7 mg. per 100 c.c. to 348.3 mg. No after-effects were noticed.

April 11, 1920.—Dog 2, white female, 16.3 kg., showed no symptoms during or after the injection of 20 gm. urea, while the blood urea rose from 24.6 mg. to 161.4 mg. per 100 c.c.

April 15, 1920.—Dog 2 received 80 gm. urea. Nausea and salivation occurred after 40 gm. urea were injected; dyspnea and vomiting after 70 gm. Blood urea rose from 22.7 mg. to 491.4 mg. Urine urea was 1,780 mg. per 100 c.c. at the end of the experiment. The animal seemed well the next morning.

April 26, 1920.—Dog 2, white female, weighing 16.6 kg.; previously injected.

4:30 p. m.—Normal blood urea, 18.4 mg.; temperature 102.2 F.

4:50-5:17 p. m.—Injected 65.3 gm. urea (Merck). At 20 gm. dog was panting markedly (240 per minute); at 40 gm. salivation, nausea and vomiting, with dyspnea. Temperature 102.0 F.

5:30 p. m.—Blood urea, 536.4 mg.

5:47-6:15 p. m.—Injected 60 gm. urea.

5:50 p. m.—Dog quiet; respiration, 14 (per minute); heart, 66.

6:00 p. m.—Dog grunting; respiration, 48; heart, 102; temperature, 101.4 F.

6:30 p. m.—Urine bloody, no casts; 5,090 mg. urea per 100 c.c. urine.

6:55-7:15 p. m.—Injected 77 gm. urea. Total injected 202.3 gm.

7:01 p. m.—Dog dyspneic (144 resp.); very restless and irritable.

7:15 p. m.—Blood urea, 1,812 mg.

7:25 p. m.—Generalized tremors.

7:30 p. m.—Temperature, 97.2 F.; tonic, then clonic convulsions, affecting the whole body, lasting thirty minutes; then spasmodic jerking of the head and limbs, with paddling movements of the forelimbs for at least three hours. Frequent urination and bloody diarrhea; the animal was unconscious, the reflexes exaggerated, the breathing jerky and rapid.

9:00 p. m.—Temperature below 94 F.

April 27, 1920.—8:00 a. m. The dog still has jerky, opisthotonic movements of the head and neck, is in deep coma with slow respiration and slow pulse, no corneal reflexes, dilated pupils.

10:00 a. m.—Similar condition; deep coma; slow twitchings. Died between 11 and 12 a. m.

1:00 p. m.—*Necropsy.*—The whole small intestine was deeply injected; the mucosa of the stomach and small intestine was swollen, hemorrhagic and necrotic; the colon hyperemic; the kidneys large, tense, bluish with the cut surface pale and swollen, with fine grayish streaks in the lower half of the cortex, the medulla glistening; the heart with the right ventricle dilated, with subendocardial petechiae; the lungs collapsed, not crepitating, slightly boggy, multiple subpleural hemorrhages; the liver dark and full of blood; the gall-bladder distended with thick tarry bile, the brain and cord hyperemic and wet; the urinary bladder empty.

Histologic examination showed marked fatty degeneration (Sudan III frozen sections, hematoxylin as counter stain) in the limbs of Henle's loops and in the collecting tubules, with cloudy swelling of the convoluted tubules; central congestion and fatty degeneration in the liver; hyperemia of the intestinal mucosa, with mucoid degeneration of the epithelium and marked edema of the submucosa; areas of edema in the lungs, with subpleural hemorrhages.

Heart's blood urea at necropsy, 1,680 mg.

Bile urea at necropsy, 1,782 mg.

April 19, 1920.—Dog 3, brown female, 9 kg., received 115 gm. urea (under ether anesthesia) and developed generalized tremors, respiratory paralysis and death within fifteen minutes after the injection. Blood urea rose from 21.8 mg. to 1,125.6 mg. The necropsy findings were similar to those in Dog 2, but with hemorrhages in the stomach alone, and no visible changes in the liver. Bile urea at necropsy was 1.560 mg. per 100 c.c.; urine urea, 2.670 mg.

June 14, 1920.—Dog 10, brown female, 8.9 kg., received 120 gm. urea. Marked salivation, restlessness, tremors, tonic and clonic convulsions, opisthotonos, nystagmus, dyspnea, diarrhea, subnormal temperature, bloody urination and death resulted. Blood urea rose from 24.2 mg. to 1,644 mg. Urine urea rose to 3,230 mg. Necropsy showed the usual findings, very similar to those in Dogs 2 and 8. Heart's blood urea, 1,702 mg.; bile urea, 1,728 mg.

April 26, 1920.—Dog 6, brown and black female, weighing 13.9 kg.

4:10 p. m.—Temperature, 103.4 F.

5:15 p. m.—Normal blood urea, 21.5 mg.

5:40-6:30 p. m.—Injected 105 gm. urea in toto.

5:48 p. m.—After 15 gm., dyspnea (180 per minute); heart, 96.

5:54 p. m.—After 30 gm., vomiting of yellow mucus; respiration, 30; heart, 96.

6:03 p. m.—After 45 gm., visible peristalsis; temperature, 101.6 F.

6:05 p. m.—After 50 gm., salivation of a thick, viscid, stringy, mucous saliva, with 412.8 mg. urea.

6:15 p. m.—After 70 gm., deep labored breathing (48 per minute); heart, 180; rapid horizontal nystagmus, salivation, marked prostration.

6:18 p. m.—After 80 gm., vertical nystagmus, dyspnea, tremors of the limbs.

6:23 p. m.—After 85 gm., slight tremors of the head and neck; tendency to opisthotonos.

6:25 p. m.—After 90 gm., respiration, 144; dog almost chokes with thick mucus; stringy saliva pours from mouth.

6:30 p. m.—After 105 gm., backward jerkings of the head.

6:32 p. m.—Generalized clonic convulsions for one minute; animal in coma; corneal reflexes absent.

6:34 p. m.—Clonic convulsions for a few seconds; then sudden cessation of heart beat and respiration, with death. Temperature, 104.8 F.

6:50 p. m.—Blood urea at necropsy 669.5 mg.; urine urea, 3,690 mg. per 100 c.c.; bile urea, 1,260 mg.; gastric contents, 1,236 mg. urea per 100 c.c.; intestinal contents, 734 mg. urea (per 100 c.c.).

Necropsy showed some edema in the lungs; hemorrhages (submucous) in the stomach; hyperemia of brain and cord; the kidneys large, tense, bluish and dry on section, with grayish, cortical streaks.

Histologic examination showed marked fatty degeneration in the cortical rays, with cloudy swelling of the convoluted tubules of the kidneys; slight fatty changes in the liver, and a patchy congestion and edema of the lungs.

May 6, 1920.—Dog 7, black female, weighing 16 kg.

4:15 p. m.—Temperature, 104.8 F.; urine, 2,470 mg. urea per 100 c.c.; blood urea, 23.6 mg.

4:50-5:52 p. m.—Injected 94 gm. urea. At 45 gm., nausea, salivation, dyspnea; at 45 gm., vomiting; at 68 gm., much vomiting; respiration, 20; heart, 72; temperature, 101.2 F.

6:03-6:45 p. m.—Seventy-nine gm. urea injected. Total injected, 173 gm.

6:10 p. m.—Temperature, 101 F.; respiration, 24; heart, 66. At 136 gm., dog's mouth full of viscid, foamy saliva; jerky grunting respiration; at 170 gm., vomiting and several times thereafter.

7:25 p. m.—Blood urea, 1,452 mg.

7:30 p. m.—Temperature, 99 F.

8:30 p. m.—Temperature, 95.4 F.; dog restless, dyspneic, cannot stand, in stuporous state, conscious only of strong stimuli; died between 9 p. m. and 8 a. m.; found very rigid.

May 7, 1920, 11 a. m.—Necropsy showed hyperemia and hemorrhages (submucous) in the small intestine, pylorus, and the upper part of the colon; the left kidney large, flabby, cyanotic, with light gray streaks in the cortex; the right kidney half the size of the left, with knobby surface, depressed scars and adherent capsule, firm on cutting, with a large white scar in the pyramids and fibrous patches in the cortex; the heart with subendocardial hemorrhages in the right auricle; the lungs edematous, especially in the lower lobes and posteriorly, with bloody frothy fluid in all the bronchi; the brain and cord hyperemic and wet.

Histologic examination showed marked fatty degeneration in the cortical rays with cloudy swelling of the convoluted tubules of the kidney, scars of chronic interstitial nephritis in one kidney with hyaline casts; hypostatic congestion and edema in the lungs.

Blood urea at necropsy, 1,539 mg.; urine urea, 3,870 mg. per 100 c.c.; bile urea, 1,242 mg.; intestinal contents, 1,836 mg. urea.

May 17, 1920.—Dog 8, white female, weighing 11.5 kg.

4:03 p. m.—Normal blood urea, 11.9 mg.

4:03-5:08 p. m.—Injected 130 gm. purified urea.

[In order to eliminate ammonium salts as a possible cause of the symptoms, a quantity of urea (Merck's) was purified by recrystallization from alcohol, so that the solution of urea was practically neutral and less than 1 mg. ammonia nitrogen per 10 gm. urea could be obtained on aeration of a 33⅓ per cent. urea solution. Ordinary urea contained, on the average, 10 mg. free ammonia nitrogen per 10 gm. urea.]

4:20 p. m.—Temperature, 102. 6 F.; heart rate, 96; respiration, 14. At 30 gm. urea, salivation; at 60 gm. urea, nystagmus; dog seems depressed.

4:40 p. m.—At 75 gm. urea, very thick stringy saliva, containing 474 mg. urea; the animal's head was thrown back in opisthotonos.

4:50 p. m.—Urea, 90 gm.; slow backward jerkings of the head.

4:53 p. m.—Urea, 97 gm.; slight tremors of the head, marked opisthotonos and nystagmus.

4:56 p. m.—Urea, 102 gm.; slight generalized tremors, snapping movements of the jaws; temperature, 102.2 F.

5:02 p. m.—Urea, 116 gm.; marked generalized tremors; increased reflex irritability.

5:04 p. m.—Urea, 120 gm.; heart rate, 84; respiration, 20; shallow. Coarse twitchings and marked opisthotonos.

5:08 p. m.—Urea, 130 gm.; dog released; tries to stand, but has clonic convulsions and falls to the floor.

5:10 p. m.—Convulsions again; the animal rolls his head and body over and over, pants rapidly for one and one-half minutes, then tremors appear.

5:12 p. m.—Convulsions again for one-half minute; heart rate, 122; the animal goes into convulsions on being touched; convulsions and opisthotonos for three minutes following the insertion of a thermometer into the rectum; then sudden cessation of convulsions, with marked panting respirations and flaccid extension of the limbs.

5:20 p. m.—Temperature, 105.2 F.; respiration, 204; heart, 66 and weak.

5:33 p. m.—Blood urea, 1,242 mg.; dog breathing jerkily; in constant tremor and opisthotonos.

5:40 p. m.—Temperature, 102 F.

5:50 p. m.—Respiration, 78; heart, 66; temperature, 100.4 F.; generalized tremors; corneal reflex now present.

6:20 p. m.—Urinated 150 c.c. bloody urine; 3,730 mg. urea per 100 c.c.

6:55 p. m.—Temperature, 98.4 F.; respiration, 28; heart, 66; tremors.

7:45 p. m.—Temperature, 98 F.; no tremors; animal in stupor.

The animal died between 8 p. m. and 8 a. m., May 18, 1920.

May 18, 1920, 1 p. m.—Necropsy showed the small intestine hyperemic, with marked hyperemia and submucous hemorrhages in the stomach, duodenum and upper jejunum; the urinary bladder empty; the kidneys bluish, with injected cortical vessels and grayish streaks in the boundary zone; the liver pale and grayish on section; the heart with subendocardial hemorrhages in the left ventricle; the lungs with marked congestion and edema, and extensive petechial and purpuric extravasations under the pleura; the brain and spinal cord hyperemic.

Histologic examination showed very marked fatty degeneration in the cortical rays, with cloudy swelling of the convoluted tubules of the kidney; some peripheral fatty changes in the liver; hypostatic congestion and edema of the lungs.

Heart's blood urea at necropsy, 1,155 mg.; bile urea, 978 mg.

May 20, 1920.—Dog 9, brown female, 10.7 kg., with large goiter, received 118 gm. repurified urea and developed nystagmus, generalized twitchings, clonic convulsions, opisthotonos, dyspnea, bloody diarrhea, stupor, subnormal temperature, death; very similar to the picture described in Dog 8. Blood urea rose from 20.2 to 1,776 mg. Urine urea was 3,010 mg. at the end of the injection. Necropsy findings were similar to those in Dog 8, but with less marked lung involvement, fatty changes in the central parts of the liver lobules, and marked nodular and cystic goiter.

Heart's blood urea, 1,362 mg.; gastric contents' urea, 1,923 mg.; bile urea, 1,422 mg.

May 16, 1920.—Dog 5, black female, weighing 12.5 kg.; normal blood urea, 18.5 mg. The right ureter was doubly ligated, under ether anesthesia. Blood urea after operation, 22.5 mg.

May 24.—Blood urea, 21.3 mg.; the wound is superficially infected, the stitches loose; some were removed. The dog weighs 11.15 kg.

May 30.—Dog weighs 11.9 kg.; wound granulating well. Blood urea, 17.2 mg.

4:30 p. m.—Respiration, 24; heart, 126; temperature, 103 F.

4:40-5:25 p. m.—Urea, 87 gm. (Daigger), injected.

4:55 p. m.—At 30 gm., respiration, 24; heart, 104; temperature, 102.8 F.

5:00 p. m.—At 40 gm., signs of nausea.

5:07 p. m.—At 55 gm., ineffectual attempts at vomiting.

5:15 p. m.—At 70 gm., respiration, 28; heart, 100; temperature, 102.8 F.

5:18 p. m.—At 75 gm., tremors of the head and forelimbs; backward throwing of the head.

5:25 p. m.—At 87 gm., sudden vomiting, with tonic and clonic convulsions, opisthotonos, nystagmus; urinated 75 c.c. pale cloudy urine, containing 1,690 mg. urea per 100 c.c. The animal tries to get up, and keeps rolling his head over and over; paddling movements of the limbs.

5:33 p. m.—Convulsions cease for a few seconds, but begin again, lasting two minutes.

5:46 p. m.—Respiration stopped suddenly.

5:48 p. m.—Heart stopped beating.

6:15 p. m.—Necropsy showed the laparotomy wound to be clean on the peritoneal surface with a few adhesions; the distal end of the right (ligated) ureter was adherent to the small intestine; the proximal end dilated (1 cm.) and the pelvis much distended with pale, yellowish fluid so that the right kidney is twice the normal size, showing hemorrhagic necrotic areas in the medulla. Many white streaks in the cortex extending to the capsule as small solid pin-head sized projections; capsule not adherent; the left kidney somewhat enlarged, cyanotic, with light lower zone in the cortex; the lungs entirely collapsed; a few hemorrhagic areas in the pylorus and duodenum; the liver dark and full of blood; the brain and cord hyperemic.

Heart's blood urea, 978 mg.; bile urea, 1,185 mg.

Histologic examination showed fatty degeneration in the limbs of Henle's loops, cloudy swelling in the convoluted tubules of the left kidney, with the tubules dilated and containing protein material; hemorrhages and necrotic areas, and evidences of an ascending suppurative pyelonephritis in the right kidney; some fatty changes in the liver.

May 27, 1920.—Dog 4, white female, 12.8 kg., had the right ureter ligated. Blood urea rose from 20.8 to 23.8 mg.;

June 3.—Sixty-three gm. urea were injected, causing nausea, generalized tremors, tonic and clonic convulsions, nystagmus, dyspnea, diarrhea and stupor, from which the animal recovered, markedly depressed. Blood urea rose from 27.8 to 1,110 mg. Urine urea was 1,900 mg. at the end of the injection.

June 4.—The dog is depressed. Blood urea, 265.5 mg.

June 10.—Seventy-four gm. urea were injected, causing a picture similar to that noted June 3, with, again, recovery of the animal. However, the convulsions were much milder than previously. Blood urea rose from 20.1 mg. to 933 mg. Urine urea was 2,040 mg.

June 11.—The dog seems well. Blood urea, 106.8 mg.

June 14.—Blood urea, 13 mg.

June 26.—Blood urea, 24.7 mg. The left ureter was ligated, the bowel being perforated accidentally. Blood urea rose to 272.4 mg. The dog was much depressed and vomited frequently, dying on the third day. Necropsy showed a suppurative peritonitis, pyohydronephrosis on the right side, some hydronephrosis on the left side with necrosis of the kidney tissue, hypostatic congestion and edema of the lungs. Heart's blood urea, 356.7 mg.; bile urea, 369.3 mg.; gastric contents' urea 448.2 mg.

July 26, 1920.—Dog 14, brown female weighing 13.2 kg.; blood urea, 18.8 mg. The left ureter was ligated. Blood urea after operation, 30.6 mg.

July 31, 2:10 p. m.—Blood urea, 19.9 mg.; some wound infection; respiration, 36; heart, 150; temperature, 106.4 F.

2:15-2:53 p. m.—Injected urea, 75 gm.

2:30 p. m.—At 30 gm., respiration, 36; heart, 168; temperature, 105.8 F.

2:43 p. m.—At 56 gm., some nystagmus.

2:53 p. m.—At 75 gm., respiration, 36; heart, 36; temperature, 104.8 F. Some retraction of the head.

2:55-3:10 p. m.—Injected 30 gm. urea. Total injected, 105 gm.

3:02 p. m.—Increased irritability; nystagmus, contracted pupils, slight tremors of the head and neck, with twitchings of the forelimbs.

3:07 p. m.—Coarse twitchings of the head and neck, with opisthotonos.

3:08 p. m.—Tonic convulsions of the head, neck and forelimbs.

3:10 p. m.—More marked tonic convulsions, flexion of the head, bulging dilated eyes; the animal tries to stand.

3:15 p. m.—Generalized tonic convulsions.

3:14 p. m.—Opisthotonos, paddling movements, clonic convulsions, alternate flexion and retraction of the head.

3:17 p. m.—Convulsions continue; the animal defecates.

3:18 p. m.—Marked clonic convulsions, with opisthotonos, rolling movements, dyspnea with puffing out of the cheeks, diarrhea—lasting for two minutes.

3:26 p. m.—Paddling movements; opisthotonos.

3:30 p. m.—The animal is much quieter, but still has coarse tremors of the head and neck, with occasional paddling movements.

3:40 p. m.—Blood urea, 1.096 mg.

3:50 p. m.—The animal is panting; has much thick, mucus saliva; marked tenesmus and diarrhea.

8:00 p. m.—Animal is very much depressed and in a drowsy state.

August 1, 9:00 a. m.—Dog found dead.

Necropsy showed some omental adhesions around the wound; subserous hemorrhages in the small intestine, with submucous hemorrhages in the stomach and duodenum, and marked hyperemia and edema of the rugae of the colon; the left kidney one and one-half times larger than the right, very soft, with numerous clusters of yellowish pinpoint areas on the surface of the kidney, alternating with red raised areas, marked hemorrhages in the pyramids and cortex, with yellow streaks in the cortex and necrotic grayish areas in the medulla; the right kidney slightly enlarged, having a boiled appearance, with grayish streaks in the lower half of the cortex; the liver pale, with darker centers; the lungs collapsed and airless, with little fluid, not much subpleural hemorrhage; the brain and cord unchanged.

Histologic examination showed, in one kidney, the usual cloudy swelling and fatty degeneration; in the other kidney, an ascending suppurative process with hemorrhagic infarcts, necrosis and hemorrhage in the medulla and masses of organisms; some fragmentation of the myocardium; necrosis of the mucosa of the colon, with marked submucous edema; subpleural hemorrhages in the lung. Heart's blood urea, 1,224 mg.; bile urea, 1,263 mg.; gastric contents' urea, 1,863 mg.

July 29, 1920.—Dog 15, black and white female, 6.7 kg., had the right ureter ligated. Blood urea rose from 23.2 mg. before, to 29.1 mg. after, operation.

August 5.—Blood urea, 30.6 mg.; 75 gm. urea were injected, producing tremors, tonic and clonic convulsions, dyspnea, nystagmus, depression and death within sixteen hours. Blood urea rose to 1,275 mg.; urine urea to 1,730 mg. Necropsy showed the usual fatty changes in the kidneys, with necrosis of the convoluted tubules in one kidney, hemorrhages in the intestine, and some pulmonary congestion and edema. Bile urea, 1,659 mg.; gastric contents' urea, 1,768 mg.

June 23, 1920.—Dog 11, white and brown female, 6.2 kg., had both ureters ligated and cut. The dog lived less than thirty-six hours, showing dyspnea and depression. Blood urea rose from 22.6 mg. to 123 mg. Necropsy showed yellowish streaks and hemorrhagic areas in both kidneys, hemorrhages and a patch of bronchopneumonia in the lungs; microscopically, fatty changes in the liver and kidneys, with necrosis of the convoluted tubules. Heart's blood urea, 243 mg.; urine (in pelvis) urea, 709 mg.; bile urea, 273 mg.; gastric contents' urea, 350 mg.

June 30, 1920.—Dog 12, white female, weighing 6.1 kg.

7:30 p. m.—Blood urea, 29.5 mg. Both ureters were ligated and cut; the dog vomited after the operation.

9:30 p. m.—Blood urea, 32.6 mg.

July 1, 2:30 p. m.—The animal seems to be in good condition. Temperature, 102.4 F. Blood urea, 92 mg.

July 2, 9:00 a. m. The dog is in fair condition, but eats very little. Temperature 101.6 F. Blood urea, 198.7 mg.

July 3, 10:00 a. m.—The animal is depressed and drowsy. Temperature, 100 F. Blood urea, 234.7 mg.

8:00 p. m.—The animal is very much depressed and drowsy. Temperature, 99.6 F. Blood urea, 366.9 mg.

10:30 p. m.—The animal is lying down in the cage, apparently in very poor spirits.

July 4, 9 a. m.—The dog is very depressed and unresponsive. Temperature, 100 F. Blood urea, 432.3 mg.

July 5, 9:00 a. m.—The dog was found dead.

Necropsy showed some stitch abscesses in the laparotomy wound, but with the peritoneal surfaces perfectly clean; both kidneys slightly enlarged, the capsules strip readily, the medulla pale, with the cortex showing grayish-yellow streaks in its lower half and, in one kidney, a few small hemorrhages; both kidneys of a brown color; the liver dark and bloody on section, with the centers of the lobules very dark in color; the stomach and the upper part of the small intestine with circumscribed areas of necrosis; the lungs collapsed anteriorly, with boggy purplish areas posteriorly and subpleural hemorrhages in some of the lobes; the brain and cord unchanged.

Histologically, the kidneys showed granular degeneration and necrosis of the convoluted tubules, with abscesses in some areas, hemorrhage, albumin precipitate in the glomerular spaces, fatty degeneration of the cortical ray tubules, and dilatation of some of the convoluted tubules; the intestine and stomach showed areas of necrosis of the villi and glands, respectively, with some submucous edema; the liver showed some central congestion, while in the lungs, hypostatic congestion, edema, and areas of subpleural hemorrhage were seen. Heart's blood urea, 329.1 mg.; urine (from pelvis) urea, 605 mg.; gastric contents' urea, 606.9 mg.; bile urea, 469.5 mg.

July 7-8, 1920.—Dog 13, yellow and white female, 6.5 kg., had both ureters ligated and cut. The animal became much depressed and died within eighty-four hours. Blood urea rose gradually from 26.9 mg. to 415.5 mg. Necropsy showed the usual fatty changes and hemorrhages in the kidneys, some hemorrhage in the duodenum, congestion and edema of the lungs. Heart's blood urea, 502.8 mg.; bile urea, 538.5 mg.; gastric contents' urea, 626 mg.; urine (in pelvis) urea, 780.8 mg.

SUMMARY

1. A series of three experiments was carried out on two dogs, demonstrating the rapid diffusion of urea into the tissues. Hence, within only a few minutes after the injection of 20, 30, 40, or 80 gm. urea, in divided doses of from 10 to 15 gm. at a time, the blood urea content was found to be very much what might be expected from the relationship between the weight of the blood in the body and the weight of the body as a whole. Thus, in a dog weighing 9.5 kg. the injection of 30 gm. urea gave a blood urea of 348.3 mg. per 100 c.c.; in a dog weighing 16.3 kg., 20 gm. urea gave 161.4 mg., 40 gm. urea gave 327.3 mg., and 80 gm. urea gave 491 mg. urea per 100 c.c. With the injection of from 30 to 40 gm. urea or more, salivation and vomiting were produced, but no other effects of any moment.

2. A second series of five experiments on five dogs was carried out to determine the toxicity of urea. The urea was injected practically continuously at the rate of 2 gm. urea (in 33⅓ per cent. solution) per minute. The average normal blood urea content of all the dogs used

was 20.7 mg. per 100 c.c.—the extremes being 11.9 and 29.5 mg., respectively.

In Dogs 2, 3, 6, 7 and 10, weighing 16.6, 9.0, 13.9, 16.0 and 8.9 kg., respectively, 202.3, 115, 105, 173 and 120 gm. urea, respectively, or, in other words, 1.2, 1.3, 0.75, 1.1 and 1.3 per cent., respectively, of the total body weight, an average of 1.1 per cent. were required to produce toxic symptoms such as generalized tremors and convulsions, coma and death. Hence, in order to produce serious symptoms, leading to death, one must inject, intravenously, at least 1 per cent. of the dog's weight in grams of urea. This confirms the results reported by Marshall and Davis.[45]

In this group of experiments there were rather broad variations in the symptoms produced, but certain symptoms were almost uniformly present. Among the earliest and most constant was salivation—of a thick, stringy, mucus type—appearing after from 25 to 50 gm. urea had been injected, and with distinct evidence of excretion of urea in the saliva. Thus, in Dog 6, after 50 gm. of urea had been injected, the thick saliva contained 412.8 mg. urea per 100 c.c.; in Dog 10, after injection of 56 gm. urea, the saliva contained 496.8 mg. urea per 100 c.c.

Vomiting was another constant symptom, usually coming on after 40 gm. urea, or more, had been given. It varied considerably among the different dogs, and was usually productive of gastric contents and some bile tinged mucus. It is interesting to note that in the only case (Dog 7) in which no convulsions were seen, vomiting was a very prominent symptom, and in this dog was seen the only example of chronic nephritis, affecting only one kidney but with very marked fibrosis.

In all the dogs of this series, after a sufficient amount of urea had been injected, increased reflex irritability, with nystagmus, tremors beginning in the head and neck and spreading downward, coarse twitchings, opisthotonic retraction of the head, tonic then clonic convulsions (except in Dog 7), unconsciousness, marked dyspnea, coma, diarrhea, and a gradual fall in temperature and pulse rate appeared in the order given, and lasted from a few minutes to many hours, most of the animals dying within twelve hours or less after the injection, two (Dogs 3 and 6) dying suddenly from respiratory paralysis. Bloody urination was rather a constant feature, the urine containing from 2,670 to 5,090 mg. urea per 100 c.c. While the picture presented by the animals varied somewhat in the relative predominance of its various elements, it could not be mistaken for anything but urea intoxication. If, after hours of twitchings and irregular panting respiration, with deep stupor and profound prostration, the animal recovered consciousness, it was very much depressed, weak, almost unable to stand, and

would lie in the cage totally unresponsive to ordinary stimuli, as if in a deep sleep, yet conscious.

The blood urea, determined about thirty minutes before or after the end of the injection, varied, of course, ranging from 669.5 mg. (rather low) in Dog 6 to 1,812 mg. in Dog 2, an average of 1,327 mg. urea per 100 c.c.

The necropsy findings in this series were strikingly uniform, consisting chiefly of marked hemorrhages in the submucosa and mucosa of the stomach and small intestine, with necrosis of the mucosa in some areas, subpleural hemorrhages with varying amounts of hypostatic congestion and edema of the lungs, and very marked fatty degeneration of the limbs of Henle's loop, with cloudy swelling of the convoluted tubules of the kidney. Apparently, therefore, the urea acted as an endothelial cell poison, and also as a toxic agent for renal epithelium.

The heart's blood urea content, postmortem, varied from 669.5 mg. to 1,702 mg. per 100 c.c., an average of 1,383 mg. The bile urea content varied from 1,242 mg. to 1,782 mg., an average of 1,514 mg. urea per 100 c.c., indicating an excretion of the urea in the bile, as might be expected from its ready diffusibility.

3. The third series of experiments consisted of the determination of the lethal dose of repurified urea (by recrystallization from alcohol), in order to exclude the poisonous action of any foreign substance, such as ammonium salts. In Dogs 8 and 9, weighing 11.5 and 10.7 kg., respectively, 130 and 118 gm. urea, respectively, were required to produce toxic symptoms, hence, 1.1 and 1.1 per cent. of the body weight, just as with ordinary commercially pure urea. The symptoms in this series, as well as the blood, bile and urine findings, and the necropsy observations, were entirely similar to those presented in the preceding series, and need not be discussed further.

4. In the fourth series, a single ureter was ligated doubly, or ligated doubly and then sectioned between ligatures, in four dogs, the animals being allowed to recover largely from the operation and then their tolerance to urea injection estimated.

In Dogs 5, 4, 14 and 15, weighing 12.5, 10.5, 10.3, 13.2 and 6.7 kg., respectively, 87.0, 63, 105 and 75 gm. urea were required to produce toxic symptoms, similar in every respect to those discussed above. Dog 4, curiously enough, received two separate injections, went into convulsions in each case, and recovered rapidly after both injections, although her symptoms were very much the same as those of the other dogs. In fact, this was the only instance of a dog recovering after convulsions. This animal was later operated on again and the remaining ureter ligated—as will be described under the next series.

It is seen, therefore, that in this series, with only one kidney functioning, the amount of urea necessary to produce toxic symptoms varied

from 0.6 to 1.1 per cent. of the body weight, an average of 0.8 per cent., as compared with 1.1 per cent .in animals with both kidneys functioning.

The general symptoms produced in this series were similar, in every respect, to those described in Series 2, and death occurred within about the same period of time after the injection. The blood urea content, determined shortly before or after the end of the injection, varied from 933 to 1,275 mg., an average of 1,078 mg. per 100 c.c. The bile and gastric contents urea showed distinct evidences of urea being excreted in those two fluids, particularly in the latter, in which the urea content averaged 1,815 mg. per 100 c.c. in two dogs. The urine urea, after the injection, varied from 1,690 to 2,040 mg. urea per 100 c.c.—distinctly low when compared with Series 2 and 3.

The necropsy findings in this series were, on the whole, quite similar to those in Series 2 and 3, except that the kidney corresponding to the ligated or divided ureter showed some hydronephrosis or pyonephrosis, of a moderate degree, with necrosis of, and hemorrhages into, the kidney tissue.

5. In the fifth and last series, four dogs were studied as to the effects of bilateral ligation and section of the ureters. These animals, Dogs 4, 11, 12 and 13, lived from three to four days, except Dog 11, which lived less than thirty-six hours. Unfortunately, not one of these animals was seen in the act of dying, so that while convulsions were never seen to occur, they may have taken place shortly before death. However, the absence of an early rigor mortis speaks against convulsions. In general, the symptoms were chiefly those of anorexia, a gradually falling temperature, an increasing drowsiness, and a marked depression, which was very obvious and constant. Irregularities in respiration appeared, but were not very constant. The blood urea rose steadily, reaching from 243 to 502.8 mg. per 100 c.c., an average of 383.7 mg. per 100 c.c. The bile urea varied from 273.0 to 538.5 mg. per 100 c.c., an average of 412.6 mg. per 100 c.c.; the gastric contents urea ranged from 350.0 to 626.0 mg., an average of 507.8 mg. per 100 c.c., indicating a distinct excretion of urea.

The necropsy findings in this series, of course, were those of degenerative changes in the kidneys, with a mild hydronephrosis, except in Dog 4, in whose right kidney (the right ureter was ligated long before the left) a very extreme hydronephrosis was present; also a suppurative peritonitis in the same animal. Dog 11 had a bronchopneumonic patch in one lung. The animals showed some hypostatic congestion and edema of the lungs, with a few subpleural hemorrhages. The stomach and intestines, in this series, were relatively unaffected, due, perhaps, to the low urea content of the blood as compared with that in the animals quoted in the second, third and fourth series.

CONCLUSIONS

The injection of urea, intravenously, in dogs produces a train of symptoms entirely analogous to that found in the convulsive or true uremia in man. Lesions are produced in the alimentary mucosa that may be related to uremic colitis. There seems to be a rather definite correlation between the severity of the symptoms and the concentration of urea in the blood.

Chronic uremia in man, ending ultimately in convulsions and coma, may be accounted for by urea intoxication, if we assume that the time element in the clinical cases is as important as the high concentration of urea in the animals, when injected. Obviously, more work is necessary, with particular reference to the use of such an apparatus as the Woodyatt machine for long continued injections of relatively low concentrations of urea. It is not implied here that only one factor is at play in the clinical forms of uremia, but urea retention must play a much more important rôle than that of an inert, harmless, waste product.

The asthenic condition produced by the ligation of both ureters involves probably many more factors than urea retention alone, and cannot, from the data at hand, be explained on any definite basis, except that such sudden occlusion of the ducts of one of the most important excretory systems must be followed by severe general metabolic disturbances.

Evidence was obtained that there is an active excretion of urea by the stomach, bile and intestine when excessive amounts are present in the blood.

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Objects and Method of Diet Adjustment in Diabetes

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In the dietetic management of diabetes we are engaged in the effort to correlate symptoms and signs shown by the patient with the kinds and quantities of food he consumes. The success of treatment, the average of results in all types of cases, depends on the truth of our concept of the relationship existing between symptoms or signs and the food supply. During the last few years the average of results obtained in the dietetic management of diabetes has been improved greatly through the work of Allen and Joslin, and the system they have developed is in some respects more logical and less empirical than any we have had heretofore. Yet the literature of the subject is still confused by a lack of unanimity among all writers as to the best manner of handling all cases. In a recent monograph, Falta [1] has again told the merits of his "cereal cure" (Mehlfrüchtekur), and endorsed methods of management that differ materially from that which has found so much favor in this country. Newburgh and Marsh,[2] failing to achieve practical results by their application of the principles of "total dietary restriction" and "undernutrition," resort to low protein high fat diets with striking success in the management of seventy-four cases. In the past, good results were obtained and may still be obtained in some cases by old fashioned "rigid" diets. The remarkable improvements that have sometimes been seen with the institution of a Donkin "milk cure," a von Duering "rice cure," a Mossé "potato cure," a von Noorden "oatmeal cure" or any one of several analogous procedures cannot be denied and have never been explained fully to the extent that one may predict with certainty just when one of these empirical procedures will and when it will not produce a result better than that attainable by a more systematic method. The present study was made in the effort to correlate some of these varying views of different writers, and, if possible, to clear away some of the confusion that they tend to create in the mind of the physician as to what method of procedure he may best follow in any specific situation.

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*From the Otho S. A. Sprague Memorial Institute Laboratory for Clinical Research, Rush Medical College. Read before the Association of American Physicians, Atlantic City, May, 1921.

1. Falta: Die Mehlfruchtekur bei Diabetes Mellitus, Berlin, 1920.
2. Newburgh and Marsh: Arch. Int. Med. **25**:647 (May) 1920.

Discrepancies in the clinical literature of diabetes arise from three main sources: (1) Confusion in the minds of writers as to the exact nature of the anomaly of the metabolism which characterizes diabetes. (2) A general tendency among clinicians to think of the food supply of the body too exclusively in terms of the diet, to the neglect of endogenous factors, and (3) the almost universal custom of thinking of the food supply simply as so much carbohydrate, so much protein, so much fat and so many calories without further analysis.

It will be necessary to develop each of these points separately.

(1). NATURE OF THE DIABETIC ANOMALY AND PRINCIPLES OF TREATMENT

(*a*) One single specific defect characterizes diabetes. This consists *in an inability on the part of the body to utilize as much glucose as may be utilized by the normal body when the supply of glucose exceeds certain limits.* The diabetic appears to be capable of utilizing a limited quantity of glucose as well as the normal individual, but fails to utilize a normal percentage of any glucose introduced into the body in excess of this limit. This limitation of the body's power to utilize glucose is present in every case of diabetes. In its absence we can not say that diabetes exists. In its presence alone, without any other accompaniment, one must say that diabetes does exist.

(*b*) The endocrine function of the pancreas, so far as we know it at all, is a single highly selective function having to do with the utilization of glucose and nothing else. All that we know of this function is deduced from our knowledge of the changes which occur in the diabetic metabolism and from the established relation between diabetes and the pancreas. Were it not for the occurrence of the metabolic phenomenon which is called diabetes, we would have no more reason to speak of a special endocrine function of the pancreas than we now have for speaking of such a function in the case of any of the organs which are not considered as endocrine organs in the usual sense of the word. No second metabolic disturbance besides diabetes has been found to occur with consistency after reduction of pancreatic substance. It is accordingly necessary to conclude that the work of the pancreas as an endocrine organ, so far as we know it today, has to do solely with the disposition of glucose, and that to lessen this work, one must lessen the quantity of glucose entering the metabolism. The pancreas is a glandular organ innervated from the celiac plexus. Vagus and sympathetic fibers pass into its substance. It is a nerve gland apparatus. Presumably it performs its function by secreting a substance having the power to dissociate the one specific sugar glucose into which all other carbohydrates that are capable of utilization on the large scale must first be converted before they can be oxidized, reduced or built up into

glycogen. That such an apparatus should be stimulated by glucose to provide for the disposition of glucose is a thought in keeping with many facts. That a weakened pancreas in the presence of a sufficient supply of glucose might be stimulated into a state of fatigue and decreased function, and that a sufficient diminution of the glucose supply might then lessen the stimulation, place the organ in a state of comparative rest, and permit it to recuperate up to a certain limit, is also quite conceivable and affords a logical explanation of many observed facts.

(*c*) The anomaly of the metabolism in which abnormal quantities of acetone, acetoacetic, and β hydroxybutyric acids appear in the tissues, blood and urine is not due directly to any impairment of the endocrine function of the pancreas. It is a secondary effect in the nature of a disturbed metabolic balance resulting from the withdrawal of oxidizing glucose, as, when one stone is withdrawn from an arch all the other members may settle to a new position. This anomaly is not peculiar to diabetes nor constantly associated with it. It occurs in other diseases. It may be made to appear in a normal subject by starvation, or a diet containing too low a proportion of carbohydrate and too high a proportion of fat, and when this is done it may be made to disappear again simply by the addition of more carbohydrate to the diet. It appears to be *the immediate result of the oxidation of certain fatty acids in the absence of a sufficient proportion of "oxidizing" (dissociated) glucose.*

There is for any given individual at any given time, *a definite ratio between the quantity of glucose oxidizing in the body and the maximum quantity of ketogenic fatty acids that can be oxidized in the same time without the appearance of abnormal amounts of the acetone bodies.* In other words, *the quantity of oxidizing glucose fixes an upper limit to the quantity of ketogenic fatty acid that can be completely oxidized at the same time.* As to the absolute magnitude of this ratio, and the degrees of its variation in different individuals in health and disease, it is too early to make a final statement. Some years ago, on the basis of chemical studies by Ciamician and Silber, and test tube experiments with acetoacetic acid, I suggested a certain type of reaction as the basis of "antiketogenesis" in which one molecule of acetoacetic acid would react with one molecule of an alcohol or glucose.[3] Zeller,[3a] working with normal individuals on ample diets consisting of carbohydrate and fat with very low protein contents, shifted the proportions of fat and carbohydrate without changing the total calories and saw acetone appear when the ratio of carbohydrate calories fell below 10 per cent. of the total, that is, when the ratio of *fat* to *carbohydrate in*

3. Woodyatt, R. T.: J. A. M. A., **55**:2109 (Dec. 10) 1910.
3a. Zeller: Arch. f. Physiol., 213, (1914).

the diet in grams was about 4 to 1. Recalculating Zeller's experiments, Lusk estimated the relative quantities of sugar and higher fatty acid that might have been oxidizing together in the body. Allowing for the formation of sugar from the glycerol of the fat, and of glucose from the protein catabolized, and for some glucose from glycogen, but not for ketogenic amino-acids from protein, Lusk suggested that possibly one triose molecule was necessary for the complete oxidation of one of higher fatty acid, that is, one molecule of glucose to two molecules of higher fatty acid, or 3 gm. to 1 gm. P. A. Shaffer [4] has worked with test tube experiments and with diabetic individuals in which he conducted metabolism studies, including observations on the respiratory quotient at the time acetone first appeared. Shaffer calculated the ketogenic acids of protein on the basis of the quantities of leucin, tyrosin and phenyl alanin found by analysis in 100 gm. of ox muscle protein by Osborne, assuming that each molecule of these known acetone formers may yield one molecule of acteoacetic acid, or its equivalent. As a result of his work, Shaffer suggests that one molecule of glucose is necessary for the complete oxidation of one molecule of acetoacetic acid, or one molecule of any higher fatty or amino-acid that yields one molecule of acetoacetic acid (or equivalent). The molecular weight of glucose being 180, of oleic acid 284, of palmitic acid 256, and the average of the two acids 270, the ratio found by Shaffer, if expressed in grams would be about 1.5 gm. higher fatty acid to 1 gm. glucose. Working with diabetic patients on maintenance diets under conditions that made it probable that the proportion of food stuffs in the diets corresponded fairly with those actually catabolized in the body, and estimating the glucose and fatty acid as hereinafter indicated, we have also observed at the time acetone appeared, ratios of 1.5 or a little above or below this figure with considerable frequency in harmony with the work of Shaffer. Accordingly, even though it may prove necessary to correct this figure as data accumulate, and for individual cases, it would seem that for clinical purposes one will make no gross error if it is assumed that the ratio of higher fatty acids to glucose, which if exceeded will lead to acidosis, is likely to be close to 1.5 to 1. (in gm.). This refers to the materials actually catabolized and to the diet only under the stated conditions.

TREATMENT. It follows from the foregoing that the rationale of dietetic management in diabetes is *to bring the quantity of glucose entering the metabolism from all sources below the quantity that can be utilized without abnormal waste; and to adjust the supply of fatty acids in relationship to the quantity of glucose* so that in the mixture of food

4. Shaffer, P. A.: Proc. Am. Soc. Biol. Chem. Fifteenth Annual Meeting, December, 1920. p. 6; J. Biol. Chem. **46**:98 (March) 1921.

stuffs oxidizing in the body, the ratio of the ketogenic fatty acids to glucose shall not exceed limits compatible with freedom from ketonuria. When, as, and if, under these conditions of relative rest for the pancreas, the glucose using function improves, then the food supply may be increased gradually in so far as this can be done without disturbing the above relations.

(2). ENDOGENOUS FACTORS OF FOOD SUPPLY. ILLOGICAL DIET RESTRICTIONS

When a man fasts he does not, of course, cease to produce heat. Normal men during a fast on light exertion have been observed to produce from 29 to 30 calories per kg. of body weight daily. For a 50 kg. man this would mean 1,500 calories per day. During the first four days of a fast, Cetti produced on the average 29 calories per kg. for a total of 1,618 calories per day, and catabolized 85.88 gm. protein for 329.8 calories and *136.72 gm. fat for the remaining 1,288 calories.*[5] In a case studied by F. G. Benedict,[6] on the second day of fasting there were produced 1,768 calories, or 29.9 calories per kg., and the individual was estimated to have catabolized 74.7 gm. protein, *147.5 gm. fat* and 23.1 gm. glycogen. These figures are by no means the highest that might be selected. Thus, a well nourished normal man weighing 50 kg., who during a fast produces 1,500 calories per day, may actually catabolize in the neighborhood of 75 gm. protein, 125 gm. fat and a little carbohydrate from glycogen. These well known facts are repeated simply to emphasize the magnitude of the food supply from the tissues in fasting, and to point out, in particular, that in fasting over 100 gm. fat may be thrown into the metabolic stream and catabolized daily. It has further been shown that the amount of fat in the fasting organism materially affects the amount of protein burned. In a critical review of the literature of the subject, Lusk has said: "Where there was much fat present, little protein was consumed; when there was little fat, much protein burned; and when there was no fat, protein alone yielded the energy for life." In a normal individual, the ingestion of fat will not prevent the death of the organism because there is a continual loss of tissue protein from the body which finally weakens some vital organ to such an extent that death takes place. *But the ingestion of fat may spare tissue fat and thus prevent the protein loss from becoming abnormally great.* It may be said that the ingestion of fat spares the individ-

5. Citation from Lusk, Elements of the Science of Nutrition, Ed. 3, 1917, pp. 86-89.

6. Benedict, F. G.: The Influence of Inanition on Metabolism, 1907, p. 194, Table 128.

ual any such protein loss as will occur if the tissue fat is allowed to become too much depleted. In this sense, the ingestion of fat by an emaciated individual spares protein for that individual. Voit found in a fasting animal that the ingestion of suitable amounts of fat scarcely influenced the protein metabolism. To one dog "which in starvation burned 96 gm. fat, Voit gave 100 gm. fat with the result that it burned 97 gm." The fat ingested simply burned instead of the body fat, but the total amount of protein and fat burned remained the same" (Lusk).

Now, if a certain diabetic patient during a fast reacts essentially as a nondiabetic individual in the same state of nutrition; and if he weighs 50 kg., produces from 1,250 to 1,500 calories, and in doing so actually mobilizes and burns 100 to 120 gm., or more, fat, the ingestion of an equal quantity of fat should leave his metabolism in the same state as before. The supply of fat would come at one time from the tissues, at another from the diet, but the quantity thrown into metabolism—the quantity presenting itself for disposition in the cells would be the same in both cases. *If these premises are sound, why then should we ever use complete fasting for diabetes?* Granting that a diabetic patient may suffer from obesity as well as diabetes—that he may have two different metabolic defects; or granting that at times it might seem desirable to starve for other conditions besides diabetes. In such cases fasting would be rational, if it would improve the general condition. But for diabetes itself, and particularly for diabetes associated with undernutrition, why for the purpose of desugarization should the patient be compelled to draw from his tissues the fat that he might draw from a diet, especially if in drawing from his tissues he lowers his fat reserves to the extent that he increases his protein losses? The striking results that have been obtained by Newburgh and Marsh with fat replacement diets bear significantly on this point. The practice of starving, or virtually starving, a patient in order to render his urine sugar "free," and then building up the diet, first with carbohydrate and then with protein, with a particular avoidance of fat, would appear to be based on the supposition that if fat were administered it would increase the catabolism of fat. But this would be in disregard of the endogenous food supply, and illustrates the necessity of thinking in terms of the metabolism rather than of the diet. As the diet falls, the endogenous supply rises to take its place, and vice versa. The lower the diet, the less its significance in calculating the food supply from all sources. It is possible to maintain the normal body with a diet that contains only 10 per cent. more calories than are produced in fasting, and the differences in the catabolism of a man when receiving no diet and when receiving a 1,500 calorie diet may be slight.

(3). DEALING WITH THE FOOD SUPPLY IN TERMS OF CARBOHYDRATE PROTEIN AND FAT

Carbohydrate, protein and fat are as such three separate and distinct substances, no one of which can be expressed quantitatively in terms of another, and if we speak of food supplies or diets as made up of so much carbohydrate, so much protein and so much fat, we simply name them in terms of three variables. Thus each particular combination or diet becomes a specific, and having learned by experience how one of them will affect a certain patient, we have no means of knowing exactly how a second dissimilar combination will affect the same patient, much less another, except by trial and experience. The number of possible combinations of three variables is infinite and the number of practical food combinations, no two of which will differ by less than 5 gm. of one ingredient or by less than 50 calories runs into the thousands. Accordingly, if we attempt to correlate symptoms and signs shown by the patient with the diet and follow the usual system of thinking of diets simply in terms of carbohydrate protein and fat, without attempting to resolve them into simpler terms, it will be necessary to establish by experiment the effects of each diet combination in every type of case. It would seem tedious and hopeless to proceed by this inductive method. A further objection to this method, and a clear advantage in using another, lies in the fact that protein, carbohydrate and fat as such are not in reality the substances that present themselves for the final oxidative attack in the body which results in the liberation of energy. These substances are resolved by the processes of digestion and intermediary metabolism into simpler substances before they can be burned in the tissues. It is not starch in the bowel nor glycogen in the liver and muscles that taxes the endocrine function of the pancreas, but the glucose into which these carbohydrates are resolved. Protein of the diet ceases to be protein and becomes a mixture of amino-acids before it can be absorbed from the bowel and these undergo deaminations, etc., prior to actual oxidation. Neutral fats, to be sure, may be absorbed in part as such, and may be deposited in the tissues as such, but before they can be oxidized and used as sources of energy, they must first be saponified into glycerol and higher fatty acids. Thus, as a matter of fact, carbohydrate protein and fat are not the actual food stuffs with which we are dealing when it comes to the final metabolic processes. In the management of the diabetic food supply, it is simpler and more rational to think in terms of the chemical metabolism. Falta devised a formula in which he added the carbohydrate of the diet to the urinary nitrogen times 2.8, or (following Lusk's suggestion) 3.65 to show the total quantity of glucose entering the body from carbohydrate and protein. Then, subtracting from this the quantity of glucose excreted in the urine, he obtained a figure for the quantity of glucose

actually utilized. The quantity excreted, divided by the total quantity, supplied gives a fraction which multiplied by 100 was Falta's diabetic quotient. The latter has a limited value. The absolute quantity utilized is of more interest. The principle is important and can be developed further.

WHAT FOODS BECOME IN THE BODY. Of the carbohydrates of the diet and tissues that play a significant rôle in heat production, all that do not already exist in the form of glucose are converted into this form by the processes of digestion or intermediate metabolism or both prior to ultimate utilization. We may, therefore, say that 100 gm. utilizable carbohydrate in the diet, if all is digested and absorbed, introduce into the metabolism about 100 gm. glucose.[7]

Fats of the diet in the course of digestion and intermediate metabolism must be saponified into glycerol and higher fatty acid before they can be oxidized. Fats such as tristearin, or an oil such as triolein, when completely saponified, yield approximately ten parts by weight of glycerol to ninety parts by weight of higher fatty acid. Glycerol is capable of conversion into glucose in the body almost gram for gram. So we may say, for clinical purposes, that 100 gm. mixed fat in the diet, if completely absorbed and catabolized, will introduce into the metabolism about 10 gm. glucose and 90 gm. higher fatty acid.[8]

Protein of the diet, or tissues, is resolved into amino-acids, and in so far as these are absorbed and catabolized, they must be deaminized (and presumably at the same time oxidized) to yield oxy- or hydroxy acids. Of these, a part is convertible into glucose, another part into β hydroxybutyric and acetoacetic acids, while a third small fraction is destroyed in as yet unknown ways. According to the experiments of Lusk on phlorhizinized dogs, which have been repeated by others, and according to numerous observations in cases of maximal human diabetes, one may say that 100 gm. mixed food protein are capable of introducing into the metabolism approximately 58 gm. glucose. The same 100 gm. of protein also introduce a certain quantity of products which are quite the equivalent of products of the higher fatty acid catabolism in that they are capable of yielding β hydroxy and acetoacetic acids. The exact quantity of these substances formed in the

7. In tables of food analyses all that is listed as carbohydrate is not necessarily utilizable carbohydrate.

8. If objection is made to the view that neutral fats may yield 10 per cent. of their weight in glucose on the grounds that ingested neutral fats have not been observed to cause the appearance of "extra" sugar in the urine of phlorhizinized dogs, it may be suggested that the ingestion of fat by a starving phlorhizinized dog would scarcely be expected to increase the fat catabolism for reasons already given unless special precaution had been taken to discharge tissue fat, etc. It is not improbable that the tissue fat that a fasting phlorhizinized dog catabolizes does produce glucose and that the glucose is then credited to protein.

catabolism of 100 gm. protein can be estimated only roughly. The amino-acids that are certainly known to yield acetone bodies are leucin, tyrosin and phenyl alanin. If we take the quantities of these amino-acids found in 100 gm. ox muscle protein by Osborne and Mendel,[9] as Shaffer has also done, and convert the weights given into gram molecules, we obtain 0.16 gm. molecules of these ketogenic amino-acids. If we assume that each of these is capable of yielding one molecule of acetoacetic, or β hydroxybutyric acid, and accept the view that one molecule of a higher fatty acid such as oleic or palmitic acid also yields one molecule of aceto-acetic (or β hydroxybutyric acid, then the 0.16 gm. molecule of ketogenic amino-acids would be equivalent in respect of its ability to form aceto-acetic acid to 0.16 gm. molecule of higher fatty acid. If the acid were oleic (molecular weight 284), this would mean 45.44 gm. The nature of this estimation is such that considerable error is scarcely avoidable, but it affords a tentative figure that serves a practical purpose, even though it may require correction in the light of experience.

It will thus be seen that all of the foods of the diet, except a small fraction of the protein, resolve themselves in the body into two things; glucose, on the one hand, and higher fatty acid (or acetone forming equivalents) on the other. If we let G stand for the quantity of glucose, and F A for the quantity of higher fatty acids that may be introduced into the metabolism by a mixture of carbohydrate, protein and fat, then

100 gm. carbohydrate yields in the body	100 gm. G	and 0 gm. FA
100 gm. protein yields in the body.....	58 gm. G	and 46 gm. (?) FA
100 gm. fat yields in the body.....	10 gm. G	and 90 gm. FA

These relationships may be expressed in the form of simple equations in which G is the total quantity of glucose introduced into the body by a given food combination; F A the total quantity of higher fatty acids (plus ketogenic amino-acids expressed in terms of higher fatty acid) C, carbohydrate; P, protein; and F, fat (neutral); thus

$$(1)\quad G = C + 0.58\ P + 0.1\ F$$

$$(2)\quad F\ A = 0.46\ P + 0.9\ F$$

If the ratio of F A : G which if exceeded leads to acetonuria is 1.5 : 1, then when $\frac{F\ A}{G} = 1.5$ we derive from (1) and (2) the equation $F = 2C + .54\ P$, which for clinical purposes may be stated simply as

$$(3)\quad F = 2C + \frac{P}{2}$$

9. Osborne and Mendel: J. Biol. Chem. **17**:366, 1914.

DISCUSSION OF HYPOTHETICAL DIETS. Let us now consider four hypothetical diets: I, II, III and IV, and analyze them from the viewpoint of their possible effects on a certain diabetic patient weighing 50 kg. Let it be assumed that each diet is completely digested, absorbed and catabolized, and that each is sufficient to cover the maintenance requirements of the body, so that we may in discussing them waive endogenous factors. Were these diets not sufficient to maintain the body, foodstuffs derived from the tissues would add themselves to the combination actually being utilized in the body and these would have to be known and allowed for. Also if some of the fat were deposited in the tissues this would have to be allowed for.

Looking at these diets simply as so many different combinations of carbohydrate, protein and fat, and going no further, they appear to be quite dissimilar. Who could certainly judge their relative effects on a patient? Diet I is a fair example of the old fashioned "rigid" diet, with almost no carbohydrate and the protein at 3 gm. per kg. of body weight. Diet II is a high carbohydrate, low protein diet. It is the

TABLE 1.—FOUR HYPOTHETICAL DIETS

	I.	II.	III.	IV.
Carbohydrate	10	77	60	51
Protein	150	30	85	70
Fat	84	108	91	125

kind of a combination that might possibly be given in a "rice cure," or a "cereal cure." This diet would permit the patient to enjoy 350 gm.—almost a pint—of boiled rice or other cereal; or, he could have 134 gm. white bread and butter—three slices—with each of his regular meals. Diet III appears to be intermediate between Diets I and II in all respects It contains less carbohydrate than Diet II, but more than Diet I; and the fat is less than in Diet II and more than in Diet I. It contains nothing that is not contained in higher quantities by one of the other diets. Diet IV resembles Diet III but might be suspected of having a higher caloric value because of its fat. We make a rapid estimation of calories and note that Diets I, II and III each represent 1,400, while Diet IV shows about 1,609 calories. On the basis of calories alone, if any of the diets were not to be tolerated, Diet IV would be the one to suspect. Now, if in each diet we add up the grams of carbohydrate plus 0.58 of the grams of protein, plus 0.1 of the grams of fat, we find that Diets I and II are alike in that each is capable of introducing about 105 gm. glucose into the metabolism. They are not only isocaloric, but will yield the same quantities of glucose.

If we assume that the patient is capable of utilizing from 105 to 110 gm. glucose per day, he might very well tolerate either of these diets. Of the two, he would be less likely to tolerate Diet I. Coming to Diet III, and estimating the glucose equivalent G, we found it to be 118. This harmless looking isocaloric diet will surely cause glycosuria in the patient who is capable of utilizing only from 105 to 110 gm. Diet IV, with 200 more calories than any of the other three, has the same glucose equivalent as Diets I and II and will probably be borne as well as either. The question arises in respect of Diet IV as to whether the high fat is permissible or in any sense objectionable. We estimate the higher fatty acid value of this diet F A by means of formula 2. Taking 0.46 the protein + 0.9 the fat, we find 32 + 113 or 145 for F A. As stated, G is 104. Then the ratio $\frac{\text{F A}}{\text{G}} = 1.4$. *As a general rule, we have found that acetone does not appear in the urine of an uncomplicated case of diabetes, or remain permanently if present when the ratio does not exceed this, provided, that* the diet is completely absorbed, and catabolized and sufficient for maintenance, so that endogenous factors of food supply do not complicate the calculation. Accordingly, we say that the quantity and proportion of fat in Diet IV are probably not too high for complete utilization in the normal manner and that there is probably nothing objectionable about it.

This leads us to the problem of calculating the theoretical optimal diet.

ESTIMATION OF OPTIMAL DIETS. If C = carbohydrate, P = protein, F = fat, G = glucose and F A = higher fatty acids (plus ketogenic amino-acids expressed in terms of higher fatty acid), we may say—as shown above—that the quantity of glucose which any given combination of foods may introduce into the metabolism is expressed by the equation: (1) G = C + 0.58 + 0.1 F and that the quantity of higher fatty acids (and equivalents) may be expressed as (2) F A = 0.46 P + 0.9 F. When the ratio $\frac{\text{F A}}{\text{G}}$ exceeds a certain value, ketonuria develops. Assuming that this ratio is 1.5, then $\frac{\text{C} + 0.58\ \text{P} + 0.1\ \text{F}}{0.46\ \text{P} + 0.9\ \text{F}} = 1.5$, when the ratio of fatty acids to glucose is as high as it may be without ketonuria. Simplifying this we obtain F = 2 C + 0.54 P, or, simply, (3) $\text{F} = 2\ \text{C} + \frac{\text{P}}{2}$. If it is agreed that the ratio F A : G shall not exceed 1.5 and that the relationships expressed in equations 1 and 2 are as given, then to estimate the optimal food combination or diet one may use equations 1 and 3. Given the quantity of glucose that the patient can utilize completely, assign this value to G in equation 1.

Thus, if 100 gm. is the highest quantity of glucose derived from all sources that the patient can utilize, 100 gm. $=$ C $+$ 0.58 P $+$ 0.1 F. In order to secure the maximal number of calories, the diet must clearly contain every possible gram of fat (at 9 calories per gram) that the value of G and the relations expressed in 1 and 3 will permit, and consequently the lowest possible carbohydrate protein fraction (at 4 calories per gram). Also, as between carbohydrate and protein, the protein must be as low as possible and the carbohydrate as high as possible for 1 gm. carbohydrate yielding 1 gm. glucose and 4 calories provides for the normal oxidation of 1.5 gm. of higher fatty acid. On the other hand, 1 gm. protein having the same caloric value as carbohydrate yields less glucose to support fat combustion and besides this yields acetone itself. If the body weight of the patient be 50 kg. and 1 gm. protein per kg. is selected as a conservative minimum; then P becomes 50 gm. and the equation (3), $F = 2\ C + \frac{P}{2}$ becomes $F = 2\ C + 25$. We have already made G $=$ 100 gm. Now, the glucose yielded by the 50 gm. protein will be 0.58 $\times$ 50, or 29 gm., leaving 100 — 29, or 71 gm., to be distributed between carbohydrate and fat. In other words, C $+$ 0.1 F $=$ 71. From this we obtain F $=$ 710 — 10 C. But we also have from the above, F $=$ 2 C $+$ 25. So 2 C $+$ 25 $=$ 710 — 10 C, solving which C $=$ 57 gm. (57.08). Substituting this value for C in F $=$ 2 C $+$ 25 we find F $=$ 139 gm. (139.16). Then, the optimal food combination that will fulfill the conditions and relations specified is: carbohydrate, 57 gm.; protein, 50 gm.; fat, 139 gm.; calories, 1,680. Proving this diet, we find the total glucose equivalent G $=$ 57.08 $+$ (0.58 $\times$ 50) $+$ (0.1 $\times$ 139.1) $=$ 99.99 as called for. Also F A $=$ (0.46 $\times$ 50) $+$ (0.9 $\times$ 139.1) $=$ 150.24 and $\frac{F\ A}{G} = 1.50$ as required.

It is apparent, that any addition of any foodstuff to this diet will make G greater than 100. If, on the other hand, one added more fat—say, 10 gm.—and subtracted 1 gm. carbohydrate, G would remain 100 and the calories would be increased by 86, but this would make $\frac{F\ A}{G}$ greater than 1.5. The effect of changing the protein can be seen by comparing the caloric value of a series of optimal food combinations with the protein rising from 0 to 2 gm. per kg. (See Table 2).

It will be observed that for each gram of glucose that can be utilized in the body there are 18 calories in the optimal food combination as here calculated. Each gram of protein in the food supply, subtracts 3 calories from the optimal number. For mental calculation, if one knows that a patient can actually utilize a certain number of

grams of glucose, take this number times seventeen as the approximate number of calories that he will be capable of using at best without glycosuria or acetonuria if the diet is most favorably balanced. Then, knowing the patient's weight the severity or mildness of the situation becomes apparent.

REPORT OF CASE

To illustrate practical aspects of the principles discussed, the following case will be found of interest.

A Greek, aged 26, was admitted to the hospital as an emergency case. He was very weak, languid and emaciated; body weight 45 kg. There was a flush on the face, and the respirations were slightly increased when quiet in bed. The plasma carbon dioxid was 23.9 volumes per cent. by Van Slyke. The breath smelled of acetone and the urine contained large quantities of sugar and acetone bodies. He had the symptoms and signs which are commonly taken to indicate a very severe case of diabetes.

TABLE 2.—Showing Optimal Food Combinations When G = 100 Gm. (in the Equation G = C + .58 + .1 F); When FA: G = 1.5; and When the Protein Is 0, 25, 50, 75 and 100 Gm. (i.e. 0; 1.0; 1.5; and 2.0 Gm. Per Kg. for a Body Weight of 50 Kg.)

	P.	C.	F.	Calories	Difference in Calories
(1)	*0.000	83.333	166.666	1833.327	76.25 (2)-(1)
(2)	25.000	70.208	152.916	1757.076	76.26 (3)-(2)
(3)	50.000	57.083	139.166	1680.826	76.25 (4)-(3)
(4)	75.000	43.958	125.416	1604.576	76.25 (5)-(4)
(5)	100.000	30.833	111.666	1528.331	

*No. 1 is hypothetical and could only be considered as the nonprotein fraction of a larger combination.

First Stage: He was put to bed with artificial warmth and placed on a diet of 400 gm. of 5 per cent. vegetables with clear broth and plenty of water. But after four days, he still passed 32 gm. sugar in the urine with acetone and 2.3 gm. ammonia. His temperature on the fourth day ranged from 96.4 to 97.6 F.; the pulse from 56 to 60, and he was so weak that it was not deemed safe to continue the effort to desugarize at that time. This ended the first stage of management.

Second Stage: He was then given for five days a diet containing 114 gm. carbohydrate, 45 gm. protein, 15 gm. fat and 760 calories. G for this diet is 142 gm. During these five days he excreted on the average 85 gm. glucose per day, so that he was disposing of about 67 gm. glucose per day from the diet alone. His general condition improved and he was then desugarized by two days of complete fasting. Having remained sugar "free" for one day, he was then given 400 gm. vegetables of the group that contains not over 5 per cent. of carbohydrate plus 1 liter of clear broth. This diet contained 12 gm. carbohydrate, 14 gm. protein and 104 calories, and G is 20 gm. But he promptly showed sugar in the urine and was again desugarized by one day of fasting. The patient had just manifested his ability actually to utilize at least 67 gm. glucose, yet the mere feeding of a diet that could yield but 20 gm. of glucose in the body induced glycosuria.

Third Stage: The urine was now free of abnormal quantities of sugar and acetone, and the patient was given a liter of broth and 200 gm. of the fresh vegetables, which he bore without glycosuria. Then he received 400 gm. greens, then 1 egg, and so on until the diet contained 314 calories, when the urine again showed sugar. Again, his condition was such that it was unsafe to continue these tactics in the effort to maintain him in the sugar "free" state. Three weeks had passed and three successive attempts had failed, although at no time had the diet been capable of introducing into the body as much glucose as the patient had actually utilized on the 760 calories diet of the second stage.

Fourth Stage: He was given a diet that contained 92 gm. carbohydrate, 103 gm. protein, 70 gm. fat and 1,416 calories. G for this diet is 159. He was on this diet for two weeks and excreted on the average 50 gm. glucose per day. Thus, he was actually utilizing about 109 gm. glucose per day. His condition was much improved and he was then desugarized by two days of fasting.

Fifth Stage: He was now in the sixth week of management, free of glycosuria and acetonuria after his third desugarization, and the attempt was again made to build up his diet gradually. But at 780 calories glycosuria recurred. A fourth desugarization by fasting was followed by another attempt, but glycosuria developed with a diet of 1,000 calories. He was desugarized a fifth time and again broke over at 1,000 calories. Nine weeks had elapsed. He was then established on a diet of 953 calories, with which he remained sugar "free." The diet now contained 50 gm. carbohydrate, 67 gm. protein and 56 gm. fat, with G = 88. The plasma carbon dioxid was 60 volumes per cent. There was no abnormal quantity of acetone in the urine and the urinary sugar as measured by the method of Benedict, Osterberg and Neuwirth showed 454, 515, 486, 425, 494, and 400 mg., respectively, per day on six successive days (tenth week).

At 22 calories per kg., even with a subnormal basal rate, this was barely a maintenance diet and it was apparent that unless something better could be done the outlook for the patient was hopeless. Much had been written about the harmful influence of too much protein in the diabetic diet. Naunyn, von Noorden, Falta and others have emphasized the necessity of keeping the protein of the diet within limits. A new interest had been given this subject by the recent report of Newburgh and Marsh. We decided to test this idea to its limit. So the patient was placed as nearly as might be in a state of specific protein hunger.

Sixth Stage: The diet was made up exclusively of rice and butter to contain 24 gm. carbohydrate, 2.5 gm. protein, 102 gm. fat, and 1,024 calories. The calories were, therefore, a little higher than he had yet been able to receive without glycosuria. He was placed on this diet and remained sugar free with excretions of 305, 400, 370, and 392 mg. respectively, on the four following days. There was no acetonuria. Now, the diet was rapidly increased; first by additions of rice alone; then by butter and rice together. In ten days it contained 84 gm carbohydrate, 11.5 gm. protein, 162 gm. fat and 1,837 calories. The urine at no time contained acetone and during these ten days had contained 308, 359, 384, 317, 545, 335, 439, 330, 486 and 333 mg. respectively, of sugar. But the patient was not in nitrogen equilibrium. The urinary nitrogen was extremely low yet it alone was exceeding that of the diet by about 1 gm. daily. So two eggs were added to the diet which made it contain carbohydrate 84 gm. protein 25 gm. (or 0.6 gm. per kg.), fat 174 gm. and calories 2,000. On this (if the stools were neglected), the patient came into nitrogen balance.

Thus, after nine weeks of trial, the ordinary practice of undernutrition had failed to establish the patient in the nondiabetic state with more than 953 calories, whereas after two weeks of the rice-butter diet the calories had more than doubled and the carbohydrate had been increased to 60 per cent. This seemed like a miracle. The 84 gm. carbohydrate when served in the form of boiled rice made a mass of about 400 gm. which was all that the patient would eat. The contrast between the trays that now came to him and those that he had formerly had aroused many remarks in the ward. Surely, it might seem that there were merits in protein deprivation. This is the type of result that astonished von Noorden when he first used his oatmeal cure. It is the type of result that has made the reputation of the von Duering rice "cure," the Mossé potato "cure," the Falta cereal "cures." However, if we examine this diet according to the equation and take the grams of carbohydrate plus 0.58 of the grams of protein, plus 0.1 of the gram of fat, we find that G is 116 gm., and it will be remembered that in the fifth stage the patient had shown his ability to utilize 109 gm. glucose although at that time he had not remained sugar "free." He was not now doing much more. The question arose as to what would have happened had the high protein diet of the fifth stage had a value for G less than 116.

Seventh Stage: A diet was composed to contain 28 gm. carbohydrate, 118 gm. protein (2.7 gm per kg. instead of 0.6 gm.), and 160 gm. fat. Total calories, 2,024 (as against 2,000). G for this diet is 112. The patient was given this diet and remained sugar free and free of acetonuria. He remained on it for eight days when it was increased to find his tolerance limit. This was found to be 119 to 120 gm.

INTERPRETATION

Clearly, the merit of the rice-butter diet had not lain simply in its low content of protein. Four times as much protein had not been incompatible with a result apparently just as good. In retrospect it is clear that the patient had shown from the second stage on the inherent ability to utilize a goodly amount of glucose. His actual tolerance was probably higher on the last day than it was on the seventh but as early as the fourth week he had burned almost as much glucose as he ever did later, so it can not be held that the final result was simply made possible by a rising tolerance. Notwithstanding his manifest ability to burn glucose, he had repeatedly shown glycosuria on very low diets that were incapable of introducing into his body as much glucose as he was able to burn, paradoxical as it may seem. This tends to encourage speculation. Shall we say that, because of the general emaciation, the starved kidneys had become abnormally permeable to sugar, that the "renal threshold for sugar" was lowered, introducing an element

of "kidney diabetes"? Or, shall we evade the issue and say that in such cases the attempt should not be made to maintain the patient in the nondiabetic state; that such patients should be fed and allowed to run sugar until they are stronger, in spite of all that we know of the power of an excess of glucose to diminish tolerance. Shall we entertain the idea that a diabetic may burn more sugar if he is given an excess than he can if he is given just the quantity that he can utilize without glycosuria? I should say no, these ideas are based on misconceptions of the food supply of the diabetic. *It was clear in this case, from the events that followed, that the patient could be given every gram of glucose that he was capable of burning under any circumstances and still be kept from showing glycosuria, provided his food supply were suitably adjusted.*

Earlier in the paper emphasis was laid on the necessity of conceiving the food supply of the diabetic patient as coming from the tissues as well as from the diet, especially when the diet falls below the maintenance requirements of the body. In the present case, we are dealing with such a situation. All of the low diets that aroused wonder because they induced glycosuria, even though incapable of introducing into the body as much glucose as the body could burn, were diets that failed to meet maintenance requirements. Consequently, when these diets were being used, the patient was drawing a greater or smaller part of his food supply from his tissues. It is not only necessary in general to consider the supply of food from the tissues, but to consider the kind and quantities of foods that will be supplied by the tissues of the particular case in hand. When a patient is thrown back on his own tissues for food, he can only draw on the materials that are there. As emphasized by the quotation from Lusk, when in fasting the body contains much fat, much fat burns and little protein; when there is little fat in the body, little fat burns and much protein; when there is no fat, protein alone burns. But whatever the composition of the body, something burns while life persists. This patient had very little fat in his body, and the quantity of glycogen was certainly negligible. Therefore, when this patient fasted, he must have produced much of his heat from protein. He weighed 43 kg. If in fasting he had produced only 15 calories per kg., this would have totaled 645 calories, an amount of energy contained in 161 gm. protein. If he catabolized this weight of protein, he would liberate 58 per cent. of this weight, or 93 gm. glucose. Then, had he at this time the ability to burn 109 gm. glucose, the addition of 20 gm. from the diet would probably cause glycosuria. Unfortunately, in this case the basal metabolic rate and the urinary nitrogen were not actually determined during the fasting periods. But the literature contains many references to the

"azoturia" of emaciated diabetics, and there can be no doubt that in such a case as this fasting induces a marked protein loss. Thus, fasting, in the case of a sufficiently emaciated patient, is the equivalent of a pure protein ration. In another case, with plenty of fat in the body, it is quite different as much tissue fat will burn and much less protein. It was pointed out earlier that the ingestion of fat by a fasting individual who already has plenty of fat in his body has very little effect on the protein metabolism. If too much fat is ingested, it may increase acidosis and increase the protein loss but in the right quantities it simply replaces tissue fat. But, if the body fat is already depleted and if, as a result of this the protein breakdown has already become excessive, then the ingestion of fat will retard the prevailing protein breakdown and reduce it to a lower level. Thus in the emaciated individual, the ingestion of fat spares protein. In this diabetic patient, in this particular state, the feeding of enough fat should reduce the protein catabolism and thereby reduce the quantity of glucose thrown into circulation. When he was given the rice-butter diet he received at once 102 gm. fat for 918 calories, almost enough to replace his minimal fasting caloric requirements, and this put a rapid quietus on the excessive protein breakdown. The quantity of glucose from protein having thus been cut down, there was then opportunity to introduce more carbohydrate in the diet which further suppressed the protein breakdown *The principal reason why this diet operated so well was because of its content of fat and because the body was so depleted in fat at the time when it was given. The patient was in a state of extreme fat starvation.* When in the sixth stage of management the patient did equally well on a low carbohydrate high protein diet this was because the combined carbohydrate and protein of that diet introduced into the body no more glucose than had formerly come from the combined carbohydrate and protein of the rice-butter-egg combination, and because this diet also contained enough fat to prevent endogenous factors of food supply from coming into action. Thus, it will be seen how the administration of a diet in excess of maintenance requirements may under certain conditions actually reduce the quantity of glucose in circulation in the body. Were the fat dropped out of these last two diets, more glucose would be thrown into metabolism by the increased breakdown of tissue protein induced by the lowering of the diet. This, I take it, is the explanation of a certain type of glycosuria which develops with prolonged undernutrition. I have seen a patient with diabetes similar to this one who could not be desugarized at all by fasting, but who cleared promptly when enough fat had been added to the diet with no other changes. This man, in fasting, catabolized so much protein that his protein sugar overtaxed this tolerance limit.

THE LAW OF PROBABILITY APPLIED TO THE FORMATION OF FATS FROM CARBOHYDRATES[1]

BY EDGAR J. WITZEMANN

In considering the possible mechanism of the formation of fatty acids in living organisms there are certain outstanding fundamental facts which must be taken into account. Five important facts of this kind follow:

(1) The fats in plants are almost altogether synthesized from carbohydrates. The same is largely true in most animals.

(2) The carbohydrates involved are largely hexoses or their polymers, since these are most abundant. In animals glucose and glycogen alone are involved so far as is known.

(3) The fatty acids occurring in plants and animals are almost solely composed of those acids having an even number of carbon atoms in their chains.

(4) The fat stores are composed almost entirely of acids having long chains with eighteen carbon atoms.

(5) Oleic acid is the principle unsaturated acid occurring in vegetable and mineral fats. The other unsaturated acids are quantitatively of isolated or much less importance.

Taking the above facts into account two general types of hypotheses have been developed concerning the chemical mechanism of the formation of fatty acids.

A. They are built up mainly from short carbon chains (less than six).

B. They are built up mainly from units of six carbon atom chains.

In the first hypothesis (3) above, namely that the acids contain an even number of carbon atoms, has received undue emphasis. Most of the meagre direct chemical data has been marshalled in support of this type of hypothesis. Thus the

[1] Contribution from the Otho S. A. Sprague Memorial Institute, Rush Medical College, Chicago, Ill.

recent theory of Magnus-Levy[1] is based on the intermediate formation of acetaldehyde in order to explain the formation of butyric, caproic and acetic acids in liver autolysis. The sugar is thought to decompose into lactic acid which in turn gives carbon dioxide, hydrogen and acetaldehyde. Buchner and Meisenheimer,[2] Nencki,[3] H. St. Raper[4] and Euler[5] have advocated somewhat similar ideas, which were extended in some respects, especially by Euler. Ida Smedley[6] pointed out the possible importance of the condensation of acetaldehyde with pyruvic acid thus, for example:

(1) $CH_3.COH + CH_3COCO_2H \longrightarrow CH_3CHOHCH_2COCO_2H$,
(2) $CH_3CHOHCH_2COCO_2H + O \longrightarrow CH_3CHOHCH_2CO_2H + CO_2$,
(3) $CH_3CHOHCH_2CO_2H - H_2O \longrightarrow CH_3CH : CHCO_2H$.

Although the above types of hypotheses all represent reactions that can or do take place they fail in important respects to account for certain facts. For instance, why should the greater portion of fats be made up of C_{18} acids if acetaldehyde and pyruvic acid are intermediate compounds in their formation? Why should not intermediate short chain acids especially occur more largely if they are intermediate stages in the formation of the typical C_{18} acids?

In emphasizing the fact that fatty acids occurring in nature contain only an even number of carbon atoms, the other fundamental facts given above are disregarded. These facts may be graphically summarized by the statement that six carbon atom sugars give rise to eighteen carbon atom fatty acids. This statement gives emphatic emphasis to the arithmetical relation of the number of carbon atoms in the compound transformed and in the final product. Emil Fischer[7] has utilized this fact clearly in his hypothetical interpretation of the formation of fats from sugar.

[1] Arch. Anat. Phys., Phys. Abt., **1902**, 365.
[2] Ber. deutsch. chem. Ges., **43**, 1773 (1910).
[3] Ibid., **10**, 1033 (1877).
[4] Proc. Chem. Soc., **23**, 235 (1907); Jour. Physiol., **32**, 216 (1906).
[5] Pflanzenchemie, II, 212 (1909).
[6] Zentr. Physiol., **26**, 915 (1912); Jour. Physiol., Dec. (1912).
[7] Untersuchung über Kohlenhydrate und Fermente, 110 (1884–1903).

"In order to derive stearic and oleic acids, which occur combined with glycerol in most fats, from sugar, it is only necessary to assume that three molecules of the latter are joined through their aldehyde groups as occurs with formaldehyde in the synthesis of sugar. Then a molecule of 18 carbon atoms would result in which only a transposition and removal of oxygen is necessary in order to produce these acids." * *

"For palmitic acid with 16 carbon atoms, which also occurs abundantly in fats, the same explanation would be adequate if one molecule of hexose and two molecules of pentoses which occur so abundantly in plants were brought together; moreover, it is also possible that it is formed from a system with 18 carbon atoms by splitting." * * * "If the sugar molecule is not completely broken up in the formation of fat, which I consider improbable, one may expect that the sugars with different carbon content will give rise to differently constituted fats."

Little or no laboratory experimental data is at hand to support this hypothesis but some of the biological data seems ummistakably clear in its support. The transformation of sugars and carbohydrates of unripe oil seeds into fat[1] appears to be quite direct. The results of Gierke's experiments[2] give even clearer support. He found in guinea pigs that fat tissue which normally is free from glycogen on the second and third days, when they are heavily fed on carbohydrates, contains much glycogen on the eighth day. In fasting or in extended feeding periods the glycogen disappeared from the fat cells by the fourteenth day.

However, this hypothesis, even in the absence of definite chemical data, commends itself for its simplicity and it was therefore of interest to learn whether there are any other considerations that can be enlisted in its support.

If fatty acids are built up two carbon atoms at a time, as suggested by the first hypothesis, it follows that since the over-

[1] Cf. for instance F. Czapek: Biochemie der Pflanzen, 2nd Ed., p. 742, et seq.

[2] Verh. d. pathol. Ges., 1906, 182.

whelming proportion of all fatty acids found everywhere are C_{18} acids, there must be a reason for the synthesis proceeding to this point and then stopping just there. Such a reason could be supplied by a sufficiently sharp change in some physical property that regulates the synthesis. But such a sharp change is not known. In fact the fatty acid series is one of the homologous series in organic chemistry most noted for regular and gradual transition in physical properties on moving up or down the series. In the absence of such a change or discontinuity in properties the synthesis of fatty acids by the addition of two carbon atoms at a time becomes amenable to the law of probability for any given set of chemical conditions. Thus if we accept the statistical fact of C_{18} acids being in the preponderance in fats these acids become our "bull's eye" in discussing their formation from this point of view.

Accurate information as to the quantitative occurrence of the various fatty acids is not available, but a qualitative estimate on the basis of such information as is obtainable from the handbooks, etc., gives a curve such as that represented by the solid line in Fig. I. Now, on connecting up the points which represent the relative abundance of the various acids instead of obtaining a smooth descending curve on both sides of C_{18}, the curve shows prominences at C_{12}, C_{24}, etc., which do not conform to the typical probability curve. If, however, the prominences are connected, as in the dotted line in the figure the typical probability curve is obtained. Moreover, the number of carbon atoms at the prominences are all divisible by six and this suggests the direct relation to the parent hexose sugars provided by Emil Fischer's hypothesis.

Accordingly, the data when assembled and judged in terms of the law of probability, appear to be against the first type of hypothesis and to support the second hypothesis.

Moreover by this view of it it becomes possible to explain why the major portion of fatty acids should have eighteen carbon atoms. If these acids are built up six carbon atoms at a time the first complex C_{12} is still sufficiently soluble either as the disaccharide or as the C_{12} acid to react rapidly with

another C_6 unit to give C_{18} either as the trisaccharide or the C_{18} acid. But in both cases the C_{18} compound is much less soluble, and presumably less reactive, than the smaller C_6 or C_{12} units so that higher acids would not be formed rapidly from C_{18} units. Here we have a sufficiently large change in physical properties when dealing with 6 carbon units to easily account for the non-formation of large amounts of fatty acids containing more than eighteen carbon atoms.

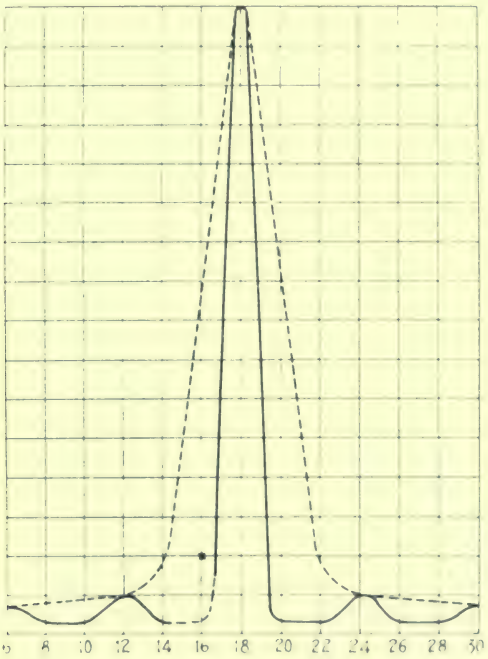


Fig. 1

The smaller occurrence of the intermediate acids, such as C_{10}, C_{14}, C_{16}, etc., may be due to the fact that they are formed from higher unsaturated acids by loss of two or more carbon atoms or by such syntheses as those involved in the first hypothesis. The apparent anomalously large occurrence of palmitic acid (C_{16}) may be accounted for by the known fact mentioned above that oleic acid undergoes a transforma-

tion at the α- and β-carbon atoms to give palmitic acid and another product. This type of reaction occurs for instance in the treatment of oleic acid with strong alkali.

In conclusion, it needs merely to be said that even the qualitative application of the law of probability appears to rationalize the problem of the mechanism of the formation of fatty acids from sugars in an interesting way. If true, the suggestion herein given constitutes a different biological application of the probability law from that known in biology as Quetelet's Law of Fluctuating Variation.

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THE CATALYTIC EFFECT OF AMMONIA ON THE OXIDATION OF BUTYRIC ACID WITH HYDROGEN PEROXIDE.

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The results described in this paper bring to light what appears to be a specific effect of ammonium hydroxide on the oxidation of butyric acid by hydrogen peroxide. The results are of such a nature that it appears that they aid in interpreting fatty acid oxidation in acidosis of the diabetic type.

The experiments here described grew out of a discordance between the author's own previous results[1] on the influence of varying amounts of potassium hydroxide on the oxidation of butyric acid with hydrogen peroxide and those of Dakin[2] on the oxidation of ammonium butyrate with hydrogen peroxide. In my own experiments it was found that the acetone yield increased as the amount of potassium hydroxide present diminished, but was never large. Dakin did not vary the amount of ammonium hydroxide used, but obtained many times as much acetone as was obtained under similar conditions with potassium hydroxide in my own experiments. Under these conditions, it seemed advisable to run simultaneous comparative experiments on the influence of ammonium and potassium or sodium hydroxides, particularly since specific effects of the salts of these bases on biological processes have become better known since the time of Dakin's publications.[3] Both Dakin's and my own previous experiments were fully confirmed. The new experiments devel-

[1] Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 83.

[2] Dakin, H. D., *J. Biol. Chem.*, 1908, iv, 77.

[3] *Cf.* Amberg, S., and Helmholz, H. F., *J. Pharm. and Exp. Therap.* 1918, xii, 19 for data and many references.

oped give additional interest to the careful experimental studies of Dakin on the oxidation of butyric acid. On the basis of the results described here, it seems not impossible that he was studying the actual mechanism of fatty acid oxidation in the diabetic type of acidosis, or at least a mechanism generically related to it.

EXPERIMENTAL.

Methods of Analysis.—When the large number of possible products of oxidation of butyric acid is considered, it is obvious that a complete quantitative analysis by the crude methods available, even for such of these compounds as are chemically known, is still out of the question. The problem, therefore, was to make such analytical determinations as would give an unmistakable indication of the state and trend of the oxidation. Suitable simple ways of determining unchanged butyric acid, acetone, and carbon dioxide were adopted and were found to give the needed information.

A portion of the oxidation solution (generally 100 or 110 cc.), with 50 cc. of water and 2 gm. of powdered manganese dioxide, was placed in a long necked distilling flask attached to a long condenser. It is necessary to use some catalyst that decomposes peroxide rapidly so that the results may be comparable, but especially to prevent the volatilization of the peroxide, which interferes with the acetone titration. A Wolff bottle connected by means of an adapter constituted the receiver. The other tubulature of the Wolff bottle was connected to two low wide mouthed wash bottles. Two wash bottles, containing sodium hydroxide and barium hydroxide, respectively, were attached to the distilling flask. During distillation a suction pump attached at the lower end circulated CO_2-free air into the apparatus and transported the CO_2 evolved on warming the mixture in the flask into the barium hydroxide solution. When only about 50 cc. remained in the distilling flask distillation was interrupted. The flask contents were filtered to remove the manganese dioxide, the filter was washed, about 50 cc. of water were added, the solution was strongly acidified with phosphoric acid, and distillation was resumed. In this way small additional amounts of CO_2 were obtained and also unchanged butyric acid as well as other volatile acids were driven over.

The CO_2 formed was determined by filtering off and weighing the $BaCO_3$ precipitated. The acetone was determined by using an aliquot portion of the distillate for the Lieben iodoform titration and the iodine consumed was calculated as acetone. This does not give the correct amount of acetone, since other iodoform-producing compounds are also present. The results, however, are satisfactory for comparative purposes.

The rapid determination of unchanged butyric acid in the distillate, even approximately, gave more difficulty until the rough method given below was adopted. The other possible volatile acids that would most likely interfere are propionic, acetic, and formic acids. Fortunately, all these acids in dilute aqueous solutions, such as were used here, have a typical form of volatility curve as was shown in another connection in a previous paper.[4] On the basis of these curves and in the absence of a better method, the attempt was made to utilize this characteristic behavior on distillation to identify the main volatile acid products and to determine them approximately at the same time.[5]

In order to determine whether this volatility curve can be used for the determination of unchanged butyric acid three distillations with known amounts of butyric acid were carried out. The amount of butyric acid given in the table was made up to 225 cc. with distilled water. Four fractions of 50 cc. were distilled off and titrated with 0.1 N NaOH.

	A	B	C
Butyric acid used, *gm.*	0.25	0.25	0.125
Fraction 1, *cc.*	10.50	10.92	5.38
" 2, *cc.*	8.04	8.08	4.08
" 3, *cc.*	5.60	5.52	2.75
" 4, *cc.*	3.30	3.20	1.61

More concordant results than these were repeatedly obtained without any special care. It will be noted that if the amount of butyric acid had been unknown in any case it could have been

[4] Witzemann, E. J., *J. Am. Chem. Soc.*, 1919, xli, 1948.

[5] Duclaux, M., *Ann. chim. phys.*, 1874, ii, 289; *Ann. Inst. Pasteur*, 1895, ix, 265. Upsom, F. W., Plum, H. M., and Schott, J. E., *J. Am. Chem. Soc.*, 1917, xxxix, 731.

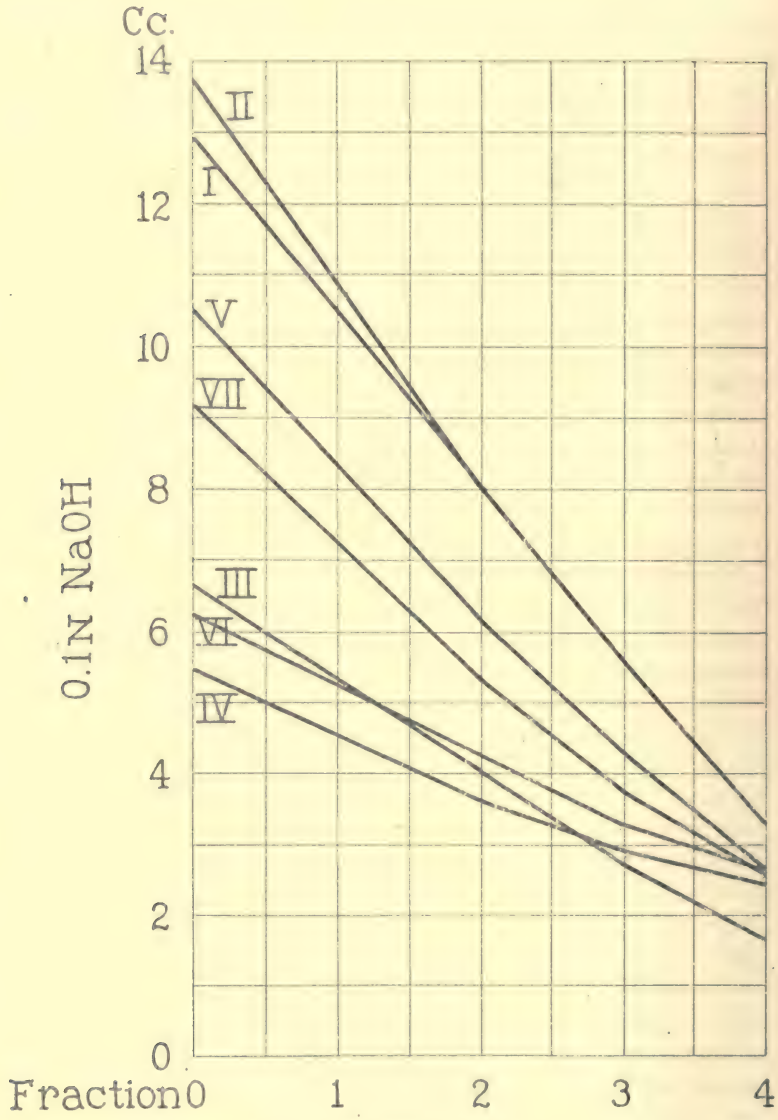


FIG. 1. Volatility curves.

determined approximately from the titration values in the other columns.

The results are given graphically in Curves I, II, and III in Fig. 1 for comparison with the curves of unknowns.

The method proved satisfactory for the purposes of this paper and was valued because the determination could be made in 15 or 20 minutes.

The data on the butyric acid recovered in such experiments are of especial importance for two reasons. (1) They enable one to determine approximately how much oxidation of all kinds has taken place. (2) Acetone is subject to oxidation in alkaline solutions with hydrogen peroxide[6] and the acetone found would not necessarily be a satisfactory index of the amount of oxidation that occurred. Owing, moreover, to the fact that enolization precedes oxidation in the case of acetone[7] the velocity of this oxidation of acetone would probably be greater when potassium is used than when ammonium hydroxide is used and so lower yields of acetone would be expected with the former. The data as determined prevent any such misunderstanding of the results.

In the preliminary qualitative experiments given below only the first distillation was carried out and no effort was made to determine the unchanged butyric acid and the carbon dioxide formed. Later the methods described above were developed and used.

It is well known that ammonium reacts in various ways with hypochlorous acid and alkali hypochlorites, hypobromites, and hypoiodites in such a way that oxidation of ammonia results. This process is apparently perceptible on adding 0.1 N iodine solution to a solution strongly alkaline with sodium hydroxide containing small amounts of ammonia. Test experiments showed that although small, the error due to such reactions in the titration of acetone was increasingly significant as the amount of acetone present diminished. This error can be avoided by adding excess phosphoric acid to the distillate containing ammonia and distilling again. Moreover, if the excess is sufficient the butyric acid is quantitatively recovered. In all experiments reported in this

[6] Witzemann, E. J., *J. Biol. Chem.*, 1918, xxxv, 89.

[7] Witzemann, E. J., *J. Am. Chem. Soc.*, 1917, xxxix, 2657.

paper 10 cc. of syrupy phosphoric acid were used in this extra distillation for the removal of ammonia.

For the sake of convenience and accuracy the butyric acid used in all experiments was converted into a 5 per cent aqueous solution so that 5 cc. corresponded to 0.25 gm. of the acid. Similar solutions of ammonium, potassium, and sodium hydroxides were prepared of which 5 cc. corresponded to 0.25 gm. of butyric acid.

Influence of Ammonium Hydroxide.—In the following experiments with mixtures of butyric acid and potassium butyrate no marked difference in the yield of acetone was observed. Aqueous solutions, 100 cc. in volume, containing 50 cc. of 3 per cent hydrogen peroxide and butyric acid as given below were allowed to stand 42 to 45 hours at room temperature and analyzed. (*a*) Contained 0.25 gm. of butyric acid as the K salt. (*b*) Contained 0.20 gm. of butyric acid as the K salt and 0.05 gm. of free butyric acid. (*c*) Contained 0.15 gm. of butyric acid as the K salt and 0.10 gm. of free butyric acid. (*d*) Contained 0.25 gm. of free butyric acid.

In all cases the iodine consumed by the distillate corresponds to about 0.001 to 0.002 gm. of acetone. In other words, the acetone formed is practically imperceptible in all cases.

A similar series of experiments was done with ammonium butyrate. The ammonium butyrate was prepared by neutralizing a butyric acid solution of known strength with ammonium hydroxide using some excess of ammonia. 100 cc. of solution contained 50 cc. of 3 per cent hydrogen peroxide and

(1)	With 0.25 gm. of butyric acid as NH_4 salt	yielded 0.0133 gm. of acetone.
(2)	With 0.20 gm. of butyric acid as NH_4 salt and 0.05 gm. of free butyric acid	" 0.0123 " " "
(3)	With 0.15 gm. of butyric acid as NH_4 salt and 0.10 gm. of free butyric acid	" 0.0053 " " "
(4)	With 0.25 gm. of free butyric acid	" 0.0041 " " "

These results show that the presence of the ammonium radical causes a marked increase in the yield of acetone. This result is in sharp contrast to what was regularly observed with potassium

butyrate as compared with butyric acid, as indicated by results such as those given above.

Another series with 0.25 gm. of butyric acid and 50 cc. of 3 per cent hydrogen peroxide in 100 cc. of solution and containing ammonium hydroxide as follows:

(5)	With 50 cc. of NH_4OH solution yielded	0.0096 gm. of acetone	
	(5 cc. = 0.25 gm. of butyric acid.)		
(6)	With 25 cc. of NH_4OH solution yielded	0.0076	" " "
(7)	" 10 " " NH_4OH " "	0.0069	" " "
		0.0075	" " "
		0.0093	" " "
(8)	" 7.5 " " NH_4OH " "	0.0075	" " "
(9)	" 6.25 " " NH_4OH " "	0.0030	" " "
(10)	" 5.00 " " NH_4OH " "	0.0021	" " "
		0.0017	" " "
(11)	" 2.5 " " NH_4OH " "	0.00138	" " "
(12)	" 0.0 " " NH_4OH " "	0.0016	" " "
		0.00153	" " "

These experiments constitute parts of three different series in which all of the reagents used and the solutions for titration were different. Although there is apparently considerable variation, the results conform with the preceding series. The results with (5), (6), and (7) suggest that apparently the maximum effect of the ammonium hydroxide is being exerted since the increase in the acetone is nowhere nearly proportional to the increase in ammonium hydroxide.

Repetition of (5), (6), and (10), brought out the fact that after 67 hours at room temperature, all peroxide had disappeared from (5), all but a trace from (6), while (10) still contained much peroxide. On distilling the mixtures in the presence of 2 gm. of manganese dioxide, the following results were obtained:

(5)	0.01887 gm. of acetone	(11.5 per cent yield).
(6)	0.0258 " " "	(15.7 " " ").
(10)	0.00523 " " "	(3.2 " " ").

By means of other experiments which will not be described here, it was found that ammonium hydroxide catalyzes the rapid decomposition of hydrogen peroxide while potassium hydroxide does not do so. The smaller amount of oxidation in (5) as compared with (6) is due to the fact that more oxygen was lost into

the air in the latter case. This simply means that the oxidation reaction is not increased at the same rate by additional ammonium hydroxide as the decomposition reaction when the concentration of the other components remains the same.

The evolution of oxygen is visible in both (5) and (6), but not in (10).

The difference in the effect of potassium hydroxide as compared with ammonium hydroxide is strikingly brought out by the following experiments in which 0.25 gm. of butyric acid and 50 cc. of 3 per cent hydrogen peroxide in 100 cc. of solution,

(13)	With 5 cc. of KOH solution (≡ 0.25 gm. of butyric acid)	yielded	0.00109	gm.	of acetone.
(14)	With 5 cc. of NH_4OH solution (≡ 0.25 gm. of butyric acid)	"	0.00126	"	" "
			0.00193	"	" "
			0.00377	"	" "
(15)	With 20 cc. of KOH solution	"	0.00092	"	" "
(16)	" 20 " " NH_4OH "	"	0.00767	"	" "
			0.00672	"	" "
			0.0338	"	" "*

* This result with the others in (16) show the extreme variations observed.

The results with (14) and (16) vary considerably with conditions, but can be duplicated for any set of conditions. Those for (13) and (15) are not subject to marked variations. Moreover, on standing longer than 2 days the results for (13) and (14) deviate farther and farther as the slow oxidation in (14) progresses. The final result in (14) after standing several weeks tends to approach the highest results obtained in (16). From this, one may conclude that the effect of ammonium hydroxide for a given time interval varies in the same way as the concentration and is in general influenced in the normal way by those factors that regulate the velocity of chemical reactions.

Interpretative Experiments.—In order to determine more accurately what happened in the above Experiments 15 and 16 with potassium and ammonium hydroxide, respectively, were repeated. After standing at room temperature nearly 3 days (16) was free from peroxide while (15) still contained unchanged hydrogen peroxide. The evolution of oxygen from both was clearly perceptible during the first day or more.

When (16) was analyzed by the method described above 0.0338 gm. of acetone or a 20.6 per cent yield was obtained. With 225 cc. or nine-elevenths of the distillate the Volatility Curve IV in Fig. 1 was obtained, which corresponds to a recovery of 0.1331 gm. or 52.4 per cent of the butyric acid used. The form of the curve shows that some lower fatty acid, probably acetic acid, was present. 0.219 gm. of barium carbonate, equivalent to 0.047 gm. of carbon dioxide or 4.7 per cent complete oxidation to carbon dioxide, was obtained. If it is assumed that all the carbon dioxide arises by oxidation of butyric acid to carbon dioxide and acetone thus: $CH_3CH_2CH_2CO_2H + O \rightarrow CH_3COCH_3 + CO_2 + H_2O$, then the carbon dioxide found corresponds to a yield of 18.8 per cent for this reaction.

The acetone obtained (20.6 per cent) and the butyric acid recovered (52.4 per cent) account for 73.0 per cent of the butyric acid used. The results indicate that much butyric acid was oxidized in the presence of ammonia.

When (15), containing potassium hydroxide instead of ammonium hydroxide, was analyzed in exactly the same way 0.00396 gm. of acetone or a 2.4 per cent yield was obtained. 0.018 gm. of carbon dioxide as barium carbonate was obtained, which corresponds to 1.8 per cent complete oxidation to carbon dioxide With nine-elevenths of the distillate the Volatility Curve V, Fig. 1 was obtained which corresponds to a recovery of 0.242 gm. or 96.8 per cent of the butyric acid used. A small amount of lower acids probably acetic was also present as indicated by the slight deviations for Fractions 3 and 4.

The total recovery of butyric acid is 96.8 + 2.4 = 99.3 per cent. The results indicate that almost no butyric acid was oxidized in the presence of potassium hydroxide.

From the two preceding experiments, one must conclude that ammonium butyrate is nearly 50 per cent oxidized under those conditions, while potassium butyrate is not appreciably attacked.

Influence of Ammonium Hydroxide in the Presence of Potassium Hydroxide.—As an interpretation of the results described above it might be suggested that in such dilute solutions the ammonium butyrate is largely hydrolyzed in comparison with the potassium butyrate and that this fact may give the key to the interpretation. In order to determine if this may be true some experi-

ments were done to test the influence of the simultaneous presence of potassium hydroxide. On this basis the amount of oxidation occurring could be expected to diminish as more potassium hydroxide was added.

Experiment 16 was repeated as follows:

(17). The same as Experiment 16 except that 5 cc. of potassium hydroxide (≡ 0.25 gm. of butyric acid) were added.

(18). The same as Experiment 16 except that 10 cc. of the same potassium hydroxide solution were added. This is double the amount of potassium hydroxide necessary to completely neutralize the butyric acid used.

The amount of ammonium hydroxide used was the same in both cases as in No. 16.

The solutions contained 1.50 per cent hydrogen peroxide at the beginning. After 92 hours No. 17 contained not over 0.06 per cent and No. 18 about 0.19 per cent hydrogen peroxide. Experiment 17 on analysis in the usual way gave 0.0239 gm. or a 14.6 per cent yield of acetone. Carbon dioxide corresponding to 0.052 gm. or a 5.2 per cent complete combustion to carbon dioxide was obtained. The fractionation of nine-elevenths of the total distillate gave the data for the unchanged butyric acid in Curve VI in Fig. 1, which corresponds to 0.152 gm. or a 60.8 per cent recovery of the butyric acid used.

No. 18 analyzed in the usual way gave 0.012 gm. of acetone or a 7.3 per cent yield. No carbon dioxide was evolved during the first distillation as with No. 20 or ammonium hydroxide experiments, but about a 6.0 per cent yield of carbon dioxide was obtained during the phosphoric acid distillation. The fractionation of four-fifths of the total distillate gave the data for the butyric acid in Curve VII, Fig. 1, which corresponds to 0.216 gm. or 86.4 per cent of the butyric acid recovered unchanged.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.
	per cent	*per cent*	*cc.*	*cc*
15	2.4	96.8	20	0
18	7.3	86.4	10	20
17	14.6	60.8	5	20
16	20.0	52.4	0	20

These results both for acetone found and for butyric acid recovered are intermediate between those of Nos. 15 and 16 as shown in the tabulated data. They show conclusively that the amount of oxidation taking place is definitely related to the amount of potassium hydroxide present when the amount of ammonium hydroxide is considerable and constant.

Influence of $NaHCO_3$ on the NH_4OH Effect. The question as to whether ammonium butyrate plays a special rôle in this reaction may be tested in another way also. In other experiments that will not be described here, it was found that the bicarbonates of potassium and sodium catalytically decompose hydrogen peroxide much faster than the hydroxides do, but not so fast as do the carbonates and ammonium hydroxide. If the effect of ammonium hydroxide is essentially due to this catalytic effect on peroxide, then considerable oxidation should take place even when the ammonium hydroxide in (16) is partly replaced with sodium bicarbonate. Three experiments were done.

(19). 5 cc. (0.25 gm.) butyric acid solution. 5 cc. (≡ 0.25 gm. butyric acid) ammonium hydroxide solution. 5 cc. sodium bicarbonate (0.028 gm. per cc. of solution). 50 cc. 3 per cent hydrogen peroxide.

(20). Same as (19) except that 10 cc. of sodium bicarbonate solution were added.

(21). Same as (19) except that no bicarbonate was used.

The results obtained on analyzing the mixtures 45 hours later are tabulated below.

No.	H_2O_2 consumed	Acetone found		Butyric acid recovered	
		gm.	per cent	gm.	per cent
19	0.46	0.0078	1.7	0.222	88.8
20	0.28	0.0078	1.7	0.245	97.8
21	1.52	0.018	11.1	0.211	84.4

These results show that although the peroxide is nearly completely decomposed in (19) and (20) there is even less oxidation than in (21) in which there was no excess of ammonium hydroxide nor any other compound catalytically active toward peroxide. Although only 58 per cent of the amount of bicarbonate equivalent to the ammonia and butyric acid was used in (19), peroxide

decomposition was nearly complete, but oxidation was very slight. This shows that the strong catalytic effect of ammonium hydroxide on peroxide is probably not the only characteristic of this compound necessary for catalyzing the oxidation of butyric acid. In this respect the behavior of ammonia seems to be specific in the same way that that of phosphate is in the case of the oxidation of glucose to carbon dioxide with hydrogen peroxide.[8] There is, however, one difference in the results as they stand and that is that the effect of ammonia can be increased in suitable mixtures with potassium hydroxide as shown in the next section, while nothing was tried that stimulates the phosphate effect similarly.

The Combined Effect of Ammonium and Potassium Hydroxide.—The interpretative experiments in the above section are defective as far as suggestiveness for biological conditions is concerned, because too much of these bases was used. The experiments described below were done in order to extend the above experiments to smaller amounts of the bases when used simultaneously. In these experiments 0.25 gm. butyric acid was used and enough potassium hydroxide to neutralize this acid (*i.e.*, 0.159 gm. of potassium hydroxide). In all cases 50 cc. of 2.3 per cent hydrogen peroxide were used. The total volume was 80 cc. The results upon analysis after 96 hours at room temperature are tabulated below.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.	H_2O_2 left.*
	per cent	*per cent*	*cc.*	*cc.*	*per cent*
22	4.0	88.0	5.0	0.0	0.908
23	17.0	57.6	5.0	5.0	0.153
24	15.0	61.6	5.0	10.0	0.151
25	11.8	68.4	5.0	20.0	0.187

* All contained 1.45 per cent H_2O_2 at the beginning.

More carbon dioxide was obtained in No. 23 than in any of the other experiments but the amount was small in all cases.

The above experiments were repeated using somewhat different amounts of alkali. Otherwise the conditions were the same.

[8] Witzemann, E. J., *J. Biol. Chem.*, 1920–21, xlv, 1.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.	H_2O used.	H_2O_2 left.*
	per cent	*per cent*	*cc.*	*cc.*	*cc.*	*per cent*
26	3.2	97.0	5.0	0.0	5.0	1.760
27	11.3	68.4	5.0	5.0	0.0	0.636
28	7.9	87.2	0.0	5.0	5.0	1.085

* All contained 1.77 per cent H_2O_2 at the beginning.

Because No. 27 contained more available alkali than the others the above experiments were repeated as follows and the results given below were obtained.

No.	Acetone found.	Butyric acid recovered.	KOH used.	NH_4OH used.	H_2O used.	H_2O_2 left.*
	per cent	*per cent*	*cc.*	*cc.*	*cc.*	*per cent*
29	2.3	91.2	10.0	0.0	0.0	1.214
30	14.8	62.8	5.0	5.0	0.0	0.293
31	12.8	67.2	0.0	10.0	0.0	0.658

* Nos. 29 to 31 contained 1.64 per cent H_2O_2 at the beginning.

The results of the three sets of experiments show: (1) That the loss in peroxide from the solution is greatest in those cases in which the most butyric acid disappeared. (2) That the presence of one equivalent of ammonium hydroxide is most favorable for oxidation when one equivalent of potassium hydroxide is also present. (3) That the presence of two equivalents of either base is not so favorable for oxidation or for peroxide decomposition as the presence of one equivalent of each. (4) That the effect of these bases is not proportional to the available alkali or the hydroxyl ion concentration.

The Relative Influence of Sodium Hydroxide upon Butyric Acid Oxidation.—In the above experiments potassium hydroxide came to be used as the alkali instead of sodium hydroxide without any reason or intention of discrimination. Normally, sodium salts are probably more abundant in biological conditions. Accordingly, it seemed desirable to do a few more experiments on the influence of sodium hydroxide upon this oxidation. In the six experiments given below 0.25 gm. of butyric acid, 50 cc. of 3.2 per cent hydrogen peroxide, and the alkali given in the table were allowed to react in a total volume of 100 cc. for 5 days at room temperature. The results are summarized in the table.

No.	Acetone found.	Butyric acid recovered.	NaOH used.	NH_4OH used.	KOH used.	H_2O used.	H_2O_2 left.
	per cent	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*per cent*
32	22.7	45.2	0.0	10.0	0.0	35	0.340
33	24.8	48.4	0.0	5.0	5.0	35	0.192
34	23.0	60.0	5.0	5.0	0.0	35	0.170
35	16.2	58.0	5.0	5.0	5.0	30	0.153
36	17.9	59.2	10.0	5.0	0.0	30	0.102
37	15.0	61.6	0.0	5.0	10.0	30	0.229

Comparison of the results in (32), (33), and (34) shows that the sodium hydroxide is somewhat less active than the potassium hydroxide when used in conjunction with ammonium hydroxide. This is confirmed by the results in (36) and (37). Other unpublished experiments on the influence of these two bases on the spontaneous decomposition of peroxide show about the same degree of difference in their action, when used in the absence of ammonia.

The results in (34) in which one equivalent of all three bases was used stand between (36) and (37), which is to be expected if the small difference in their behavior is correct.

Biologically these results are of interest because they show that if under any conditions fatty acid oxidation in the organism resembles these oxidations either the potassium or sodium salt of mixtures would be attacked with about the same ease in the presence of ammonia.

The results in this section are of especial interest in another way because they show that the catalytic influence of ammonium, sodium, and potassium as the bases is just the reverse of their influence as the phosphates, upon the peroxide oxidation of a number of simple organic compounds. Further experiments upon mixtures will be done in developing these unpublished results on phosphates.

Partial Interpretation.—The experiments in the preceding section bring out a number of facts concerning the interpretation. (*a*) The ammonium and potassium butyrates here under consideration are the salts of a weak and a strong base, respectively, with a weak acid. At these dilutions there would be considerably more free butyric acid produced by hydrolysis when

ammonia only is used. That this free acid is not the only significant factor in the catalysis is shown by the fact that the simultaneous presence of both bases promotes the greatest oxidation. (*b*) That the mere presence of many butyrate ions does not determine the oxidation, is shown by the fact that in the presence of ammonium hydroxide alone, in which, of all the experiments, there is the smallest concentration of butyrate ions, the oxidation proceeds much faster than when potassium hydroxide only is used where the butyrate ion concentration is greatest. (*c*) That the butyrate molecules do not determine the oxidation is shown by the fact that oxidation is slowest where the concentration of these is greatest; namely, in the solutions containing potassium hydroxide alone. (*d*) That the capacity to decompose peroxide spontaneously may be a factor is suggested by the fact that potassium decomposes peroxide much more slowly than ammonium hydroxide. That this is not the sole factor is shown by the experiments with sodium bicarbonate which decomposes peroxide well but does not facilitate these oxidations. (*e*) The concentration of the hydroxyl ion has no definite relation to the velocity of the oxidation as observed with these three bases.

The known facts about these oxidations are not yet sufficient for a satisfactory interpretation. The statements given in the next two paragraphs suggest a partial interpretation.

The oxidation of butyric acid in the presence of ammonia described above may belong to the so called "coupled" or induced oxidations. The formation of a peroxide of ammonia may represent one phase of this coupling. That such a peroxide of ammonia may be obtained under suitable conditions was shown by Melikoff and Pissarjewski.[1] If this peroxide is the intermediate concerned it is much more unstable than the peroxide of potassium since when equal concentrations of the two bases are placed in solutions of hydrogen peroxide the ammonium hydroxide decomposes the peroxide much more rapidly than the potassium hydroxide does. The conceptions of an ideal catalyst and the theory of dislocation of Boeseken[2] offer a concrete partial interpretation of this part of the effect of ammonia. According to this

[1] Melikoff, P., and Pissarjewski, L. [illegible] 1894, xxvi, 341.

[2] Boeseken, J., *Koninklijke Akademie van Wetenschappen te Amsterdam*, 1914, xxiii, 291; *Rec. trav. chim. Pays-Bas*, 1920, xxxix, 633.

view of catalysis that catalyst will be best which has the greatest tendency to form the least stable complex with the substrate.

If it is granted that the data justify this much of an interpretation for the interaction of ammonia and peroxide they fail to indicate how such a complex activates the oxidation of butyric acid. Sweeping hypotheses on this subject would be undesirable, especially in view of the fact that equally anomalous data are already in hand showing that when these bases are used as phosphates the effects on the oxidation of butyric acid are quite different.

Possible Biological Significance of the Ammonia Effect. — The experiments showed that butyric acid, whether free or combined with sodium or potassium, is peculiarly susceptible to oxidation by peroxide when ammonia is also present, and that under these conditions relatively little carbon dioxide but much acetone is obtained. This high degree of acetone formation depends upon the simultaneous presence and the interaction of ammonia, peroxide, and butyric acid. Without entering into details it would seem that these three components play an active rôle in metabolism, particularly in the hepatic metabolism, and that acetone as acetoacetic acid is also formed in this organ. It will be recalled, for instance, that Embden and Kalberlah[11] found that the liver is the only organ that, on perfusion in the surviving state with normal blood, gives acetoacetic acid. Moreover there is in the liver an active traffic in ammonia equivalents involved in its important deaminizing and urea-forming functions. That it can actually supply this component of this reaction system better than other organs has recently been brought out by experiments of Keeton[12] of this laboratory, who found, in harmony with old observations, that when an inorganic acid is administered by stomach to dogs it is partially excreted as the ammonium salt, and causes an increase in the urinary ammonia nitrogen, both relative and absolute. When, however, the acid is injected into a peripheral vein, although it may cause some absolute increase of the urinary ammonia, it causes little or no increase in the quan-

[11] Embden, G., and Kalberlah, F., *Beitr. chem. Physiol. and Path.*, 1906, viii, 121. Embden, G., and Engel, H., *Beitr. chem. Physiol. u. Path.*, 1908, xi, 323.

[12] Keeton, R. W., *J. Biol. Chem.*, in press.

tity of ammonia nitrogen relative to the total nitrogen. This difference in the behavior of acid administered by the portal and the peripheral routes, respectively, is interpreted as due to the fact that ammonia is an available base in the liver and enters into the neutralization of acid when the acid is neutralized in the liver, but plays little part in the neutralization of acids elsewhere in the body.

It may not seem amiss to suggest, since the same type of oxidation as that observed in the above described test-tube experiments is known to occur in the liver in a high degree, that this oxidation in the liver may possibly be favored as in the test-tube by ammonia.

The question has long been discussed as to whether the formation of acetoacetic and β-hydroxybutyric acids in the body represents a purely abnormal type of fatty acid oxidation, that only occurs under certain conditions (as in diabetic acidosis, etc.) or whether it is a step in the normal oxidative breakdown of the fatty acids, having an even number of carbon atoms, which is obscured in health because of the rapid occurrence of subsequent events. The first view would make acetoacetic acid an abnormal product, the latter would make it a normal product, which in acidosis of the diabetic type fails to break down further. Neubauer[13] in his well known study of the breakdown of the fatty and amino-acids favored the view that the formation of acetoacetic acid is a normal step.

The above experiments and discussion would tend to favor the view that the formation of acetoacetic acid may be considered as a normal step in the oxidation of fatty acids, in harmony with Neubauer's conception, and particularly in media in which ammonia is available. Moreover the special occurrence of this type of oxidation in the liver might possibly be brought into relationship with the special availability of ammonia in this organ, it being less characteristic of the oxidation of fatty acids elsewhere in the body, where relatively less ammonia is available. According to this line of thought a certain special physiological function of the body, namely its power to form acetoacetic acid, would be associated with a special characteristic of ammonia

[13] Neubauer, O., *Deutsch. Arch. klin. Med.*, 1908-09, xcv, 211.

or its equivalent and the suggestion would follow that where acetoacetic acid is formed in the body ammonia may be present and acting in this characteristic way. The problem of why in health the evidence of acetoacetic acid formation is suppressed, *i.e.* the problem of "antiketogenesis" (or "ketolysis") is clearly another matter.

SUMMARY.

Experiments were performed with the object of determining the reason for the difference in the results obtained in the oxidation of butyric acid with hydrogen peroxide in the presence of different alkaline substances.

In the presence of potassium hydroxide in amounts varying from 0.20 to 4.0 equivalents no appreciable oxidation of butyric acid took place, as was proved by the almost quantitative recovery of the unchanged acid.

In the presence of ammonium hydroxide in amounts varying from 0.20 to 10.0 equivalents much oxidation took place.

The amount of oxidation in the presence of ammonia was found to increase with increase in the ammonium hydroxide, other things being equal, until more than 4.0 equivalents of ammonium hydroxide were present, after which it decreased somewhat.

This decrease with large excess of ammonium hydroxide was due to the spontaneous liberation of oxygen by the action of ammonium hydroxide on the hydrogen peroxide, before it could be utilized in oxidation.

If one equivalent of both ammonium hydroxide and potassium hydroxide is used more oxidation takes place than if two equivalents of either of these bases are added.

The type of oxidation observed in these experiments was mainly of the β type or the conversion of butyric acid into acetone and 1 molecule of carbon dioxide. Consequently the statement in the above paragraph is equivalent to saying that the presence of ammonia in a solution containing potassium butyrate and hydrogen peroxide catalyzes the oxidation of butyric acid to acetone and carbon dioxide.

The additive effect of ammonium and potassium here described in favoring oxidation constitutes a chemical analogy to the biological effects of certain mixed salts.

The experiments on the additive effects of sodium and ammonium show that this pair acts nearly the same as potassium and ammonium. There is just enough difference in the behavior of potassium and sodium to constitute a confirmation of unpublished observations of specific effects of these bases as phosphates.

A few suggestions for a partial interpretation were made. It is clear, however, in this case, as in the phosphate effect on glucose previously reported, that the rôle of alkalinity and alkali is not primary in this oxidation. A satisfactory interpretation is particularly desirable in view of the possible biological significance of the results.

Finally in discussing the possible biological significance of these results it is suggested that the ammonia effect here described may be the agency by which the normal oxidation of fatty acids is brought about in the liver. This suggestion rests upon the fact that in the liver the substances required for this effect are all available and that this organ also normally shows the greatest tendency to form acetoacetic acid.

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AMMONIA EXCRETION FOLLOWING EXPERIMENTAL ADMINISTRATION OF ACIDS VIA THE STOMACH AND PERIPHERAL VEIN.

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The type of acidosis which attains its highest expression in diabetes is, as is well known, very frequently attended by a marked increase in the excretion of ammonia in the urine. The acids in this case (β-hydroxybutyric and acetoacetic) are in part neutralized by the organic basic ammonium. Yet the phenomenon of neutralization by ammonium is not constant in the other types of acidosis in man. It appears to play relatively a small part in the acidosis of uremia (1), even when this attains an extreme degree, and it is missed in some of the other types of spontaneous acidosis, which have been observed in man. On the other hand, the feeding of dilute mineral acids (2, 3) to man, if in sufficient quantity, leads consistently to increased excretions of ammonia in the urine comparable to those seen in the diabetic type of acidosis. However, if a man, who is excreting increased quantities of ammonia, either as an incident in the course of a spontaneous diabetic acidosis or as the result of an alimentary administration of an acid such as hydrochloric, be given a sufficient quantity of bicarbonate of sodium (4), the increased excretion of ammonia is depressed or wholly annulled. The administration of the stronger base results in the displacement of the excess of ammonia from the urine, and it would not seem irrational to compare the phenomenon with that which occurs in the test-tube, when sodium bicarbonate, magnesium oxide, or calcium oxide, are added to a watery solution of ammonium chloride, acetoacetate, β-hydroxybutyrate, or the like with the liberation of free ammonia and the formation of a new salt of the base employed. In the body such a release

of ammonia from its salts (or the prevention of its union with acids) by the action of a stronger base could be conceived as leading simply to some other disposition of the ammonium radicles displaced or spared from combination with acids. They might then, for example, enter into the synthesis of urea.

Although the phenomenon of neutralization of acids by ammonia has long attracted interest and has been spoken of by some writers (5) as one of the important compensatory mechanisms by which the body may protect itself against inroads of acid, it is a noteworthy fact, that it is not a mechanism which is called into play consistently by the human organism under all conditions, in which it might be supposed in reason that every available means of protection should be employed. A patient may die of acid intoxication developing in the course of a pyelonephritis without ever having called on this resource to a noticeable degree. In man the classical examples of neutralization of acids by ammonia are afforded in acidosis of the diabetic type. It has been reported in clinical methyl alcohol poisoning (6) which, however, under experimental conditions (7) may not always be associated with an acidosis. It is also said to develop in the acidosis of cholera (1) and it has been shown to occur in connection with certain of the diarrheal diseases of children, which in some cases, resemble closely the acidosis of diabetes. The two chief conditions, however, in which the phenomenon is known to occur regularly are those mentioned. Moreover, this type of acidosis associated with increased ammonia output is not seen in all types of animals but occurs most readily in man and the carnivorous animals, and only to a lesser extent in the herbivorous animals. These considerations suggest that the neutralization of acids by ammonium is not a mechanism of universal significance in the whole problem of neutrality regulation by living cells, but that it is rather a special phenomenon which only occurs under certain conditions.

It has been pointed out by others (8) that, in discussing observed variations in the excretion of acetone bodies in the urine, it is pertinent to consider the question of where the acetone bodies are formed. Well known evidence afforded by liver perfusion experiments, favors the view, that the liver, of all the organs, may be regarded as the largest single producer of the acetone bodies. The

liver is also commonly recognized as an organ in which the total traffic in NH_2 groups is greater than in any single tissue, even though the function of deamination and the synthesis of urea were performed to some extent elsewhere as well.[1] With its deaminizing and urea-forming function it must be considered that the liver is preeminent in respect to the total quantity of ammonia equivalents that it normally handles in the course of the day. It is generally accepted that the ammonia which in acidosis appears in the urine in the form of salts of acids represents basic nitrogenous material, which normally would be excreted for the most part in the form of urea. Also acids, which are formed and liberated in this organ which conducts the heaviest traffic in ammonia or its equivalents, are generated in a part of the body where the chances for neutralization by ammonia should be exceptionally favorable.

If it be granted that the phenomenon of neutralization of acids by ammonium has been observed with the greatest consistency and in the highest degree in two conditions: (*a*) a type of acidosis in which the acids are largely hepatogenous in origin, and (*b*) a type following the administration of acids—but that the phenomenon is found to be less marked or entirely absent in other types of acidosis the question would arise as to why the ingestion of acid should produce an effect like that of one type of acidosis and unlike that of the other types. Could this mean that in order to be neutralized to the highest degree by ammonium the free acids involved must be formed within or be introduced into the liver?

In reviewing the literature one is struck by the fact that those who have observed increased excretion of ammonia following the administration of acids have almost without exception given the acid by the alimentary route. Winterberg (10) injected rabbits subcutaneously (two experiments) with 0.25 N acid and found the ammonia, sodium, and potassium content of the urine greatly

[1] Stadie and Van Slyke (9) in their study of a case of acute yellow atrophy have recently concluded that the rôle of the liver in these processes cannot be assumed in its entirety by the rest of the body. Only in pathological conditions, which are associated with almost complete destruction of the liver tissue, does the organism fail to metabolize in a normal fashion the amino-acids even though they be presented in high concentrations.

increased. The total nitrogen was not correspondingly increased. But one of these animals died at the end of 24 hours and with the other the experiment was discontinued in about the same time. These two isolated experiments would scarcely seem sufficient to settle the point. One would have to consider that the local destructive effect on the tissues of an amount of 0.25 N acid sufficient to cause death in 24 hours is not to be neglected. Whereas, the literature contains other reports (11, 12, 13) concerning the injection of acids by vein we have not found any in which the behavior of the urinary ammonia was recorded.

The experiments to be described were undertaken for the specific purpose of comparing the relative effects on the ammonia excretion in the urine of acid administration by the alimentary route and by the peripheral vein. Alimentary administrations should subject the liver to the effects of a relatively large quantity of free acid, while the latter would afford opportunity for the neutralization of more of the acid in the blood or tissues outside of the liver.

EXPERIMENTAL.

Methods.

Female dogs of approximately 16 kilos body weight were used. They were fed a constant diet, consisting of milk 100 to 200 cc., bread 50 to 100 gm., sodium acetate 0.5 gm. twice daily at 6.30 a.m. and p.m. To the morning feeding 100 cc. of physiological salt solution were added, which served to keep the volume of liquids the same in the experimental and the control periods. The sodium acetate was given to promote uniform elimination. Collections of urine were made twice daily by catheterization and irrigation of the bladder. The cages were scrubbed and disinfected every 12 hours to prevent decomposition of occasional specimens passed spontaneously. This procedure gave consistent ammonia values. The urines were placed on ice without preservatives, the ammonia and total nitrogen being estimated shortly after the closure of the 24 hour period.

The ammonia was estimated by means of fo malin titration (Ronchese and Malfatti) as described by Wiechowski (14). The urine was well diluted and the titration was carried to the first

permanent pink. This method was checked with Folin's (15) aeration method. The practical agreement of the methods for present purposes is shown in the following protocol.

Formalin titration.	Folin method.
0.30	0.27
0.25	0.25
0.37	0.37
180 cc. of 0.1 N HCl by stomach.	
0.41	0.41
0.46	0.44
0.39	0.39
0.37	0.38
0.42	Not estimated.
250 cc. of 0.1 N HCl by vein.	
0.46	0.45
0.51	0.51
0.52	Not estimated.
0.33	0.33
0.49	0.48
0.44	0.42

The total nitrogen was estimated by the Kjeldahl method, the creatinine according to Folin (15).

On the morning of the experiment the salt solution was omitted from the diet. The acid was then administered either by stomach tube or into the leg vein. For the latter a cannula was inserted into the vein under local anesthesia, and the acid was introduced by gravity throughout the period of 1 hour.

Experiments.—Five animals were used in the studies, and seven complete (gastric and intravenous administration) experiments made, but the protocols of only two animals are reported in detail. Dog 2 died during the course of the intravenous injection, the time of which was shortened from 1 hour to 30 minutes. Dog 3 was a young, highly irritable animal, which never gave consistent results during the control period. However, the results in this case were in keeping with those reported. The dose of acid injected into Dog 4 proved to be too toxic, so that at the end of 24

hours food was refused, the nose was hot, and the quantity of urine was much reduced. However, the output of ammonia and total nitrogen during the first 24 hours was the same as for the day preceding the experiment, in agreement with the findings to be reported in detail. On the succeeding days the total nitrogen

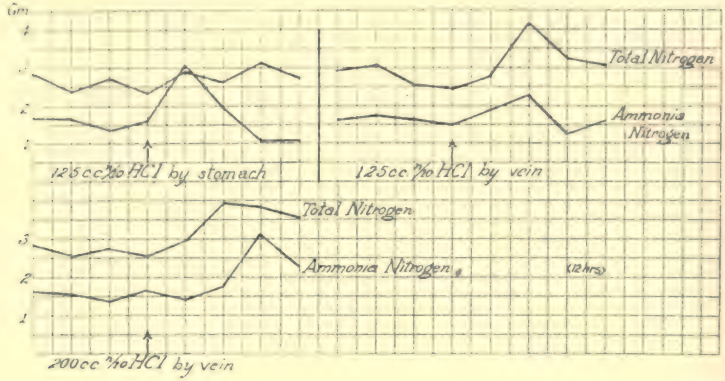


CHART 1. Changes in nitrogen excretion after acid administration in Dog 1.

In the ordinates of Charts 1 to 4 are expressed the values of total and ammonia nitrogen in grams, the latter multiplied by 10. In the abscissæ two spaces represent 12 hours. Record covers 48 hours before and after experiment.

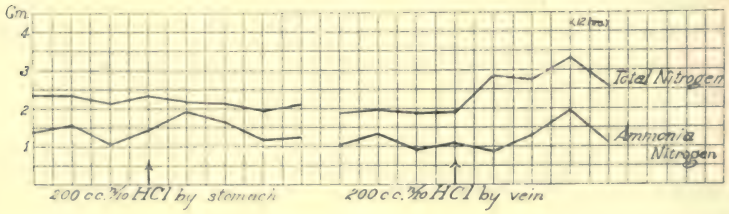


CHART 2. Changes in nitrogen excretion after acid administration in Dog 1.

fell slightly below normal, but the ammonia increased. The results of the experiments on Dogs 1 and 5 are presented in the charts. In Charts 1 to 4 the quantitative output of ammonia nitrogen and the total nitrogen, expressed in grams, are plotted for 48 hours before and after the experimental periods. Chart 5

shows the complete record of Dog 1 through two consecutive experiments. The nitrogen values here are expressed in 24 hour intervals. The figures from which the charts were compiled are to be found in Tables I to V, Chart 1 corresponding to Table I, etc.

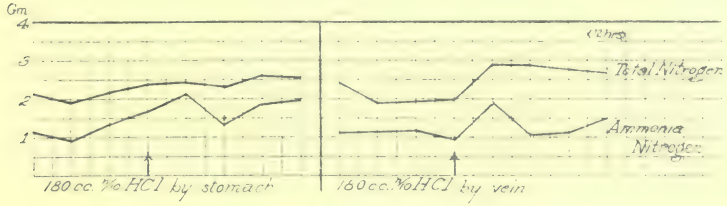


CHART 3. Changes in nitrogen excretion after acid administration in Dog 5.

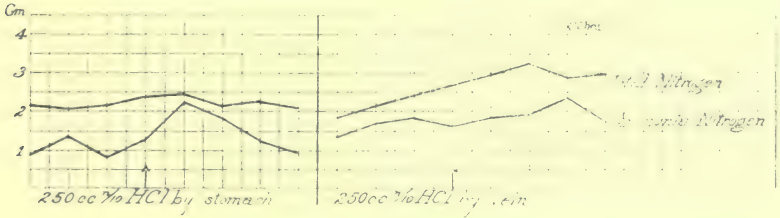


CHART 4. Changes in nitrogen excretion after acid administration in Dog 5.

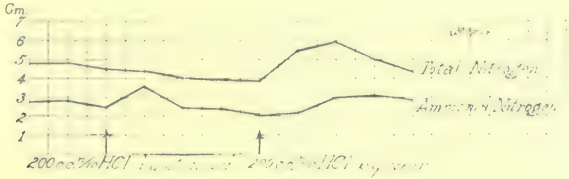


CHART 5. Changes in nitrogen excretion following successive acid administrations in Dog 1.

In the ordinates of Chart 5 are expressed the values of total and ammonia nitrogen in grams, the latter multiplied by 10. In the abscissæ two spaces represent 24 hours. All of the time (11 days) which elapsed in the course of the experiment is represented in the chart.

Ammonia Excretion Following Administration of Acid by Stomach.—These graphs show clearly an absolute increase in the ammonia nitrogen without changes in the total nitrogen. In

other words, some of the nitrogen which normally appeared in the urine in other forms (*e.g.*, urea) has been diverted to the neutralization of the acid and now appears as ammonia in the urine. This simply confirms commonly accepted facts.

Ammonia Excretion Following Administration of Acid Intravenously.—The curve of total nitrogen gradually rises. The suddenness of the rise appears to depend within limits on the quantity

TABLE I.

Changes in Nitrogen Excretion after Acid Administration in Dog 1.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1921	*gm.*	*gm.*
Jan. 18, p.m.	2.86	0.17
" 19, a.m.	2.43	0.17
" 19, p.m.	2.71	0.14
" 20, a.m.	2.35	0.16
" 20, "	Gastric administration 125 cc. of 0.1 N HCl.	
" 20, p.m.	2.93	0.30
" 21, a.m.	2.64	0.20
" 21, p.m.	3.03	0.16
" 22, a.m.	2.70	0.15
Jan. 24, p.m.	2.97	0.17
" 25, a.m.	3.03	0.18
" 25, p.m.	2.51	0.16
" 26, a.m.	2.46	0.15
" 26, "	Intravenous administration 125 cc. of 0.1 N HCl.	
" 26, p.m.	2.79	0.19
" 27, a.m.	4.14	0.29
" 27, p.m.	3.25	0.13
" 28, a.m.	3.06	0.21

of acid given. The ammonia nitrogen follows closely, so that the percentage relationships between ammonia nitrogen and total nitrogen are fairly maintained. This is interpreted to mean that there is a definite toxic destruction of protein induced by the acid injection. The results of these experiments differ from the foregoing in the maintenance of a practically normal percentage of ammonia nitrogen, which suggest simply an acceleration of normal proteolysis.

Comparison.—Tables VI and VII contrast the reactions of the body to acid administration by different routes. The values of total and ammonia nitrogen actually excreted are found in Columns a and b. The first reading of each column in the various experiments gives the excretion during the control period, the second figure, the excretion during the experimental period (24 hours following acid administration). To secure the values for

TABLE II.

Changes in Nitrogen Excretion after Acid Administration in Dog 1.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1920	*gm.*	*gm.*
Dec. 8, p.m.	2.38	0.13
" 9, a.m.	2.36	0.15
" 9, p.m.	2.16	0.11
" 10, a.m.	2.33	0.14
" 10, "	Gastric administration 200 cc. of 0.1 N HCl.	
" 10, p.m.	2.21	0.20
" 11, a.m.	2.19	0.17
" 11, p.m.	1.97	0.12
" 12, a.m.	2.08	0.13
Dec. 12, p.m.	1.92	0.11
" 13, a.m.	1.99	0.13
" 13, p.m.	1.93	0.10
" 14, a.m.	1.96	0.11
" 14, "	Intravenous administration 200 cc. of 0.1 N HCl.	
" 14, p.m.	2.85	0.09
" 15, a.m.	2.72	0.13
" 15, p.m.	3.36	0.20
" 16, a.m.	2.56	0.11

the control period the excretions of total and ammonia nitrogen were averaged for three 24 hour intervals preceding the experiment. It has been assumed for the purposes of this tabulation that the *ratio* of ammonia nitrogen to total nitrogen, established in the control period (Column c), was maintained into the acid period except in so far as it was changed by the formation of ammonium salts. That such an assumption can be made without undue violence is seen by reference to preceding data and

discussion. For it was pointed out that the gastric administration of the acid was without effect on the total nitrogen, and that in the administration by peripheral vein, the results suggest an acceleration of normal proteolysis. The ammonia nitrogen then, which is to be expected on such a basis, can be deduced by multiplying this ratio by the total nitrogen actually excreted after acid administration. Such a value is shown in Column d. The dif-

TABLE III.

Changes in Nitrogen Excretion after Acid Administration in Dog 5.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1921	gm.	gm.
Mar. 1, p.m.	2.14	0.12
" 2, a.m.	1.93	0.09
" 2, p.m.	2.12	0.14
" 3, a.m.	2.34	0.17
" 3, "	Gastric administration 180 cc. of 0.1 N HCl.	
" 3, p.m.	2.42	0.21
" 4, a.m.	2.30	0.13
" 4, p.m.	2.53	0.18
" 5, a.m.	2.51	0.20
Feb. 14, p.m.	2.44	0.11
" 15, a.m.	1.91	0.12
" 15, p.m.	1.97	0.12
" 16, a.m.	1.99	0.10
" 16, "	Intravenous administration 180 cc. of 0.1 N HCl.	
" 16, p.m.	2.86	0.19
" 17, a.m.	2.85	0.11
" 17, p.m.	2.78	0.11
" 18, a.m.	2.67	0.16

ference between this value and the quantity actually excreted (Column b) would indicate the quantity of ammonia concerned in the neutralization of the acid administered. This value has been expressed in cc. of 0.1 N NH_4OH and denominated "Extra ammonia."

It is obvious from the tables that a much larger quantity of the acid administered by the gastrointestinal route can be accounted for as "Extra ammonia," than of that given by the peripheral

TABLE IV.

Changes in Nitrogen Excretion after Acid Administration in Dog 5.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1921	*gm.*	*gm.*
Apr. 3, p.m.	2.14	0.09
" 4, a.m.	2.07	0.14
" 4, p.m.	2.18	0.08
" 5, a.m.	2.35	0.13
" 5, "	Gastric administration 250 cc. of 0.1 N HCl.	
" 5, p.m.	2.41	0.22
" 6, a.m.	2.12	0.18
" 6, p.m.	2.29	0.12
" 7, a.m.	2.09	0.09
Mar. 8, p.m.	1.82	0.14
" 9, a.m.	2.20	0.16
" 9, p.m.	2.37	0.18
" 10, a.m.	2.67	0.17
" 10, "	Intravenous administration 250 cc. of 0.1 N HCl.	
" 10, p.m.	2.94	0.18
" 11, a.m.	3.30	0.20
" 11, p.m.	2.89	0.24
" 12, a.m.	2.94	0.18

TABLE V.

Changes in Nitrogen Excretion Following Successive Acid Administrations in Dog 1.

Date.	Nitrogen excretion per 12 hrs.	
	Total.	In ammonia.
1920	*gm.*	*gm.*
Dec. 7–8	4.75	0.28
" 8–9	4.74	0.29
" 9–10	4.49	0.25
" 10, a.m.	Gastric administration 200 cc. of 0.1 N HCl.	
" 10–11	4.40	0.36
" 11–12	4.05	0.25
" 12–13	3.91	0.24
" 13–14	3.89	0.21
" 14, a.m.	Intravenous administration 200 cc. of 0.1 N HCl.	
" 14–15	5.57	0.22
" 15–16	5.92	0.30
" 16–17	5.06	0.31
" 17–18	4.30	0.28

TABLE VI.

Quantity of Acid Excreted in the Form of Ammonium Salts in the 24 Hour Urine before and after the Administration of Hydrochloric Acid by the Gastric and Intravenous Routes.

(Dog 1, female mongrel, weight 16.17 kilos.)

Experiment.	0.1 N acid given.		Nitrogen excretion per 24 hrs.				Extra ammonia as 0.1 N NH_4.†	Remarks.
	Amount.	Route.	Total.	In NH_3.	Ratio $\frac{b}{a}$	Ratio $\frac{b}{a}xy$*.		
			a	*b*	*c*	*d*		
	cc.		*gm.*	*gm.*		*gm.*	*cc.*	
1			5.43	0.32	0.058			24 hr. control (average of 3 days).
	125	Gastric.	5.57	0.50		0.32	128	24 hr. period following acid.
2			4.66	0.27	0.058			24 hr. control (average of 3 days).
	200	Gastric.	4.40	0.36		0.25	79	24 hr. period following acid.
3			5.63	0.33	0.058			24 hr. control (average of 3 days).
	125	By vein.	6.93	0.42		0.40	14	24 hr. period following acid.
4			3.95	0.23	0.058			24 hr. control (average of 3 days).
	200	By vein.	5.57	0.22		0.32	0	24 hr. period following acid.
5			5.48	0.33	0.060			24 hr. control (average of 3 days).
	200	By vein.	6.94	0.32		0.42	0	24 hr. period following acid.

* The grams of ammonia N which would be excreted if the ratio of ammonia nitrogen to total nitrogen remained the same as in the control period.

† Calculated as the absolute quantity excreted (Column b) minus the above (Column d) and converted into terms of cc. of 0.1 N NH_4 for comparison with the quantity of 0.1 N acid given.

vein. It is also noted that following gastrointestinal administration by no means all of the acid given can be recovered in the

TABLE VII.

Quantity of Acid Excreted in the Form of Ammonium Salts in the 24 Hour Urine before and after the Administration of Hydrochloric Acid by the Gastric and Intravenous Routes.

(Dog 5, female bull, weight 17.8 kilos.)

Experiment.	0.1 N acid given.		Nitrogen excretion per 24 hrs.				Extra ammonia as 0.1 N NH_4.†	Remarks.
	Amount.	Route.	Total.	In NH_3.	Ratio $\frac{b}{a}$	Ratio $\frac{b}{a}$ x y*		
			a	*b*	*c*	*d*		
	cc.		*gm.*	*gm.*		*gm.*	*cc.*	
1			4.41	0.25	0.057			24 hr. control (average of 3 days).
	180	Gastric.	4.72	0.34		0.27	50	24 hr. period following acid.
2			4.35	0.25	0.057			24 hr. control (average of 3 days).
	250	Gastric.	4.53	0.41		0.26	107	24 hr. period following acid.
3			4.10	0.21	0.051			24 hr. control (average of 3 days).
	180	By vein.	5.71	0.30		0.29	7	24 hr. period following acid.
4			4.73	0.32	0.067			24 hr. control (average of 3 days).
	250	By vein.	6.24	0.38		0.42	0	24 hr. period following acid.

* The grams of ammonia N which would be excreted if the ratio of ammonia nitrogen to total nitrogen remained the same as in the control period.

† Calculated as the absolute quantity excreted (Column b) minus the above (Column d) and converted into terms of cc. of 0.1 N NH_4 for comparison with the quantity of 0.1 N acid given.

form of ammonia. This agrees well with the recent report of Stehle and McCarty (3), who found that even with the estimation of all the bases in the urine and the increased H ion concentration

they were able in two cases (man) to account for only 96 and 61 per cent, respectively, of the acid administered.

Creatinine Excretion.—The creatinine excretion was followed in the case of Dog 5 after the administration of 180 cc. of 0.1 N HCl by mouth and 250 cc. of 0.1 N HCl by vein. The variations were so slight as to be within the range of experimental error. We may conclude then that these procedures were without effect on the output of creatine, at least, in this particular animal.

DISCUSSION.

The experiments confirm the well known observation that the administration of acid by the alimentary route to dogs leads to a definite increase in the absolute quantity of ammonia and the quantity relative to the total nitrogen excreted in 24 hours. These increases always took place within the first 24 hours following the acid administration and subsided thereafter. When the same quantity of the same acid was administered to the same dog by the peripheral intravenous route there was as a rule an increase in the total output of ammonia. However, these increases were smaller in degree than those following the alimentary administration, they tended to occur later, and were invariably associated with an increase in the total nitrogen output, so that the ratios of the ammonia nitrogen to the total nitrogen were relatively little affected. Comparing the effects of the alimentary and intravenous administrations of acid, the former were characterized by an increase of the ammonia nitrogen with relatively little increase in total nitrogen. When there was an increase in the total nitrogen at all it was late (3 to 4 days) and never pronounced. In the case of the intravenous injections, the total nitrogen was always increased more and earlier, than in the case of the alimentary administrations. Indeed the speed of the onset of this rise seemed to be determined (within limits) largely by the quantity of acid administered. The total ammonia output then rose correspondingly, but there was little or no evidence of a shift in the nitrogen partition. These results indicate that when the acid enters the body *via* the portal route more of it is neutralized by ammonium than when it enters otherwise.

These results are at variance with those reported by Winterberg (10) to which previous reference has been made. He found that

the ammonia nitrogen was increased without a corresponding increase in the total nitrogen, when rabbits (two experiments) were injected *subcutaneously* with 0.25 N acid. The cause of this discrepancy has not been investigated in detail, but as stated above one must consider that the local destructive action on the tissues of a mass of acid, sufficient to cause death in a rabbit at the end of 24 hours, is not to be neglected. That such a result as he obtained may be associated with the use of extremely toxic or lethal doses of acid is indicated in our findings in Dog 4. The complete details of the experiment are not reported since the animal refused food and showed other signs of profound intoxication. In this case there was no increase in either the total nitrogen or the ammonia nitrogen during the first 24 hours, before the signs of intoxication were manifested and while the animal was still eating. During the 3 succeeding days, with the establishment of these signs of intoxication the ammonia nitrogen increased while the total nitrogen did not increase correspondingly. Finally it must be stated that he was using rabbits while our experiments were conducted upon dogs.

The experiments seem to lend color to the idea developed in the introduction, that neutralization of acids by ammonium may be more in the nature of a localized (hepatic) function than any universal function of the body that is called upon to combat acids in general. It is suggested that when free acids are formed in the liver or are introduced into the liver, and must unite with bases, that in this particular place a larger percentage of the acids will unite with ammonia than will do so when the neutralization takes place elsewhere. Acids must be neutralized by bases that are available at the site of their neutralization. This would imply that in the liver there is ammonium available for the neutralization of acids. The fact that Gad-Andresen (16) and others have shown that the ammonia content of the liver tissue by analysis is not different from that of the heart, muscle, or fat is no argument against this view. For we are here speaking of a moving traffic in ammonia equivalents and not of a cross-section of this traffic at any one time, such as these estimations present.

Such a view would imply that, when endogenous acid appears in the urine in large quantities combined with ammonium, the acids may have been formed chiefly in the liver. Thus diabetes

with its supply of acetone bodies arising chiefly in the liver, presents the classical case of acidosis with large ammonia output. The diarrheas of Asiatic cholera and (less constantly) of infancy with their increased ammonia could be viewed as developing their acids in the portal circuit. Nephritis with its acidosis would then become a disease in which the acids concerned are not hepatogenous in origin, but in which some other principal site or sites of acid formation should be sought. This is not out of harmony with the view that the acidosis of nephritis is due wholly or in part to delayed excretion of acids formed in the body at large. A pathological condition in which the ammonia excretion is increased without a corresponding acidosis would be a condition having no direct interest in the present discussion.

CONCLUSIONS.

1. Administration of 0.1 N HCl by stomach tube to dogs causes an absolute increase in the ammonia nitrogen excreted in the urine, while the total nitrogen remains practically constant.

2. Intravenous administration of the same dose of acid to the same animal causes an increase in excretion of both ammonia and total nitrogen, but the normal ratio between these is fairly well maintained.

3. Alimentary administration of acid is associated with a shift in the nitrogen partition towards the ammonia fraction. Such a shift in nitrogen partition is absent following intravenous injections, if the dose of acid be not too toxic.

BIBLIOGRAPHY.

1. Sellards, A. W., The principles of acidosis and clinical methods for its study, Cambridge, 1917.
2. Howland, J., and Marriott, W. McK., *J. Biol. Chem.*, 1917, xxix, p. v.
3. Stehle, R. L., and McCarty, A. C., *J. Biol. Chem.*, 1921, xlvii, 315.
4. Janney, N., *Z. physiol. Chem.*, 1911–12, lxxvi, 99.
5. Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1918, xxxv, 101.
6. Król, J., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 444.
7. Haskell, C. C., Hileman, S. P., and Gardner, W. R., *Arch. Int. Med.*, 1921, xxvii, 71.
8. Woodyatt, R. T., *J. Am. Med. Assn.*, 1916, lxvi, 1910.
9. Stadie, W. C., and Van Slyke, D. D., *Arch. Int. Med.*, 1920, xxv, 693.
10. Winterberg, H., *Z. physiol. Chem.*, 1898, xxv, 202.

11. Oré, *Compt. rend. Acad.*, 1875, lxxxi, 833.
12. Guttman, P., *Arch. path. Anat. u. klin. Med.*, 1877, lxix, 534.
13. Szili, A., *Arch. ges. Physiol.*, 1906, cxv, 82.
14. Wiechowski, W., Analyse des Harns, Wiesbaden, 11th edition, 1910, 535.
15. Folin, O., Laboratory manual of biological chemistry, New York, 2nd edition, 1920.
16. Gad-Andresen, K. L., *J. Biol. Chem.*, 1919, xxxix, 267.

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CURVE OF SUGAR EXCRETION IN SEVERE DIABETES.

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The work reported in this paper represents a study of the total quantities of sugar excreted in the urine of a number of diabetic patients during periods in which they were being held in the non-diabetic status, or what is sometimes called the "sugar-free" condition, by dietary restriction, and during the periods in which the diets were gradually increased until clearly abnormal quantities of sugar appeared in the urine. The purpose has been to plot the curves of glucose excretion by diabetic patients on diets increasing gradually from those on which they show no abnormal glycosuria up to the point at which definitely abnormal glycosuria is induced. The total sugar excreted in the urine every 24 hours was determined quantitatively by the method described by Benedict and Osterberg[1] for the quantitative determination of sugar in normal urine.

The glucose equivalents of the diets were calculated as the utilizable carbohydrate plus the protein $\times$ 0.58 plus the fat $\times$ 0.1 as per the equation $G = C + 0.58P + 0.1F$[2] in which G = glucose, C = carbohydrate, P = protein, and F = fat. The total glucose supply to the organism was not always represented entirely by the diet since in most instances the diets were at times below maintenance values, necessitating the catabolism of protein and fat from the tissues. In some instances also, the quantity of fat in the diet could have exceeded the quantity actually catabolized.

Case 1.—(Miss R.) Age 60 years. Weight 38.6 kilos. On admission to the hospital she showed marked denutrition, glycosuria, acidosis, alkali deficit, and symptoms of acid intoxication. She was given alkali and

[1] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.
[2] Woodyatt, R. T., *Arch. Int. Med.*, 1921, xxviii, 125.

reduced to the non-diabetic status by dietary restriction. On a diet of 32 gm. carbohydrate, 57.5 gm. protein, and 53 gm. fat—containing 835 calories and the equivalent of 71 gm. glucose (G), the total sugar eliminated each 24 hours for 3 days remained constantly at a little over 400 mg. per day (see Chart 1). The diet was then increased very slowly; and on 35 gm. carbohydrate, 60 gm. protein, and 60.5 gm. fat—924 calories (G = 76), the sugar excreted per day for 2 days was no higher than it had been on the 835 caloric diet. On the 3rd day, however, while the patient was still on the same diet, the sugar eliminated suddenly increased to 1,050 mg. and a subsequent addition equivalent to 4 gm. glucose and 34 calories resulted

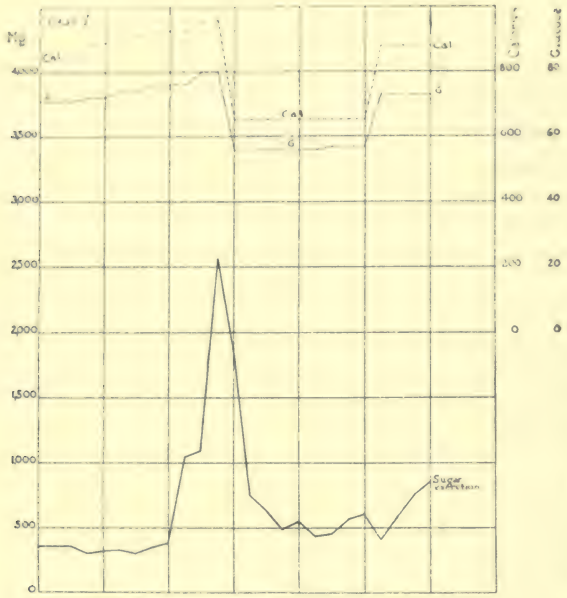


CHART 1.

in the excretion of 1,092 mg. for the first 24 hours and 2,556 mg. for the second 24 hours on which the same diet was maintained. Without entering into a discussion of the factors which may have determined this sudden acceleration of the glucose excretion in this particular case, it affords an example of the suddenness with which the sugar excretion may jump from a normal level to an abnormally high level.

Case 2.—(Mr. M.) A young man, 26 years of age, weighing 50 kilos who entered the hospital in severe acidosis and denutrition and who had been "desugarized" by a diet consisting of green vegetables and broth aggregating 298 calories (G = 27), excreted 520 mg. of sugar on the first day of observation. Thereafter, the sugar excreted in the urine remained

practically constant from day to day during the time in which the diet was increased from a caloric value of 298 to one of 1,336 calories, the G for the diet rising from 27 to 116 gm. Thus, the total quantity of sugar eliminated for the 24 hour period in which the diet contained 398 calories with $G = 51$, was 680 mg. and it was only 683 mg. on a 1,236 calorie diet with $G = 101$ gm. The next addition, which brought the diet up to 1,404 calories, and increased G to 123 gm. (an increase of 22 gm.) resulted in a total urinary sugar excretion of only 1,006 mg.; whereas, upon a further addition which increased the value of G by only 11 gm. and the calories by 50, the total

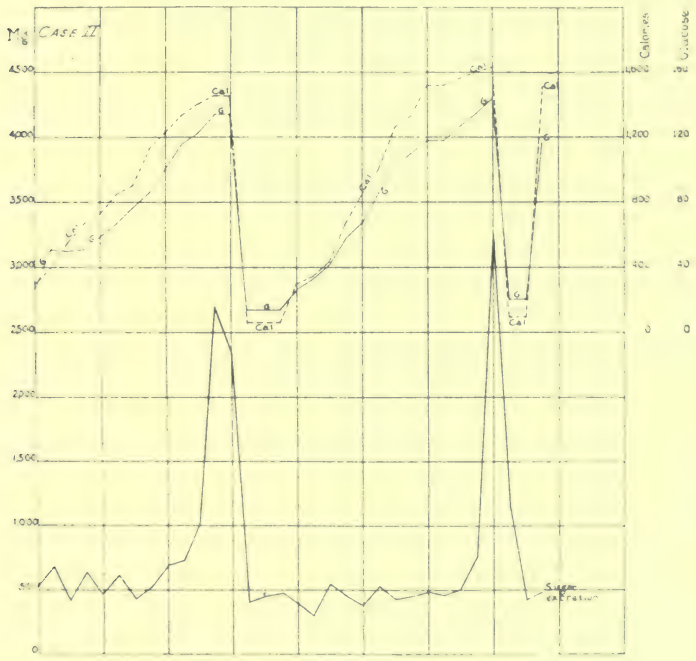


CHART 2.

sugar eliminated was 2,695 mg. Thus, increasing the calories in the diet by some 800 calories and the glucose equivalent by 89 gm. caused little or no increase of the sugar excretion, but the further addition of only 218 calories and the equivalent of 33 gm. glucose caused a critical break in the curve. On the lowest diet the calories were 298; therefore most of the food supply on which the patient was subsisting was then coming from the tissues. The highest diet barely approached maintenance requirements. Accordingly, the figures for the diet never represented the total glucose supply from all sources, and the curve of increase of the latter is not known. Yet the experiment shows a sudden transition from the normal

to the abnormal in respect to the sugar excretion. The diet was then reduced to 64 calories with *G* for the diet itself at 14; and the experiment was repeated with the same result. There was almost no variation in the total sugar eliminated per day during the time that the diet was increased from 14 *G* and 64 calories to 135 *G* and 1,601 calories; whereupon, a subsequent addition of food equivalent to only 9 gm. glucose and 36 calories, which brought *G* for the total diet to 144 gm. and the calorie value to 1,637, resulted in a sugar excretion of 3,268 mg. for that day. It was quite evident that the curve representing the sugar eliminated in the urine by

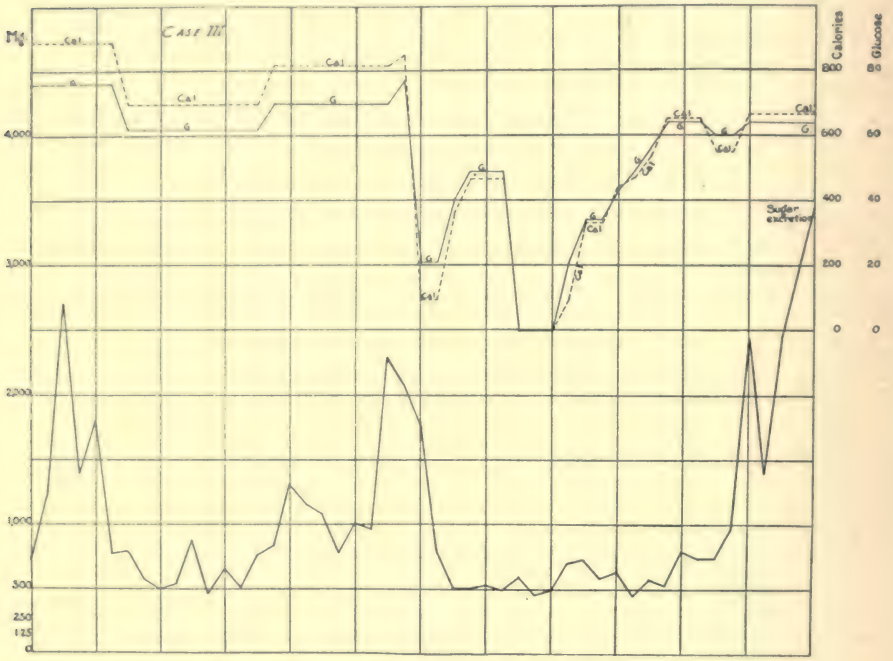


CHART 3.

days, did not follow the curve representing the gradual rise in the caloric or glucose equivalents of the diet. It remained more or less constant until the glucose equivalent of the diet reached a certain value, when there was a sudden upward break in the curve of sugar excretion. This upward break represented an absolute acceleration of the sugar excretion out of proportion to that which had resulted from all earlier additions to the diet. It also represented an even greater increase of the percentage excretion of the last increments to the diet.

Case 3.—(Mrs. F.) A young woman 24 years of age. Weight 36 kilos. Her glucose-using power was very low. On an 888 calorie diet (with

G = 76 gm.) she excreted 725 mg. sugar on 1 day, and 2 days later, while still on the same diet, excreted 2,711 mg. On absolute starvation for 3 consecutive days her urinary sugar excretion was 601, 460, and 504 mg. per day. On a 651 calorie diet, with G = 64 gm., the urinary sugar was 537, 802, and 750 mg., respectively for the 3 days on which the same diet was maintained; whereas, a slight subsequent rearrangement in the diet, increasing its calorie value only to 665, with G as before, resulted in the excretion of 2,440 mg. sugar. Here again, the transition from the "sugar-free" state to one in which the urine contained distinctly

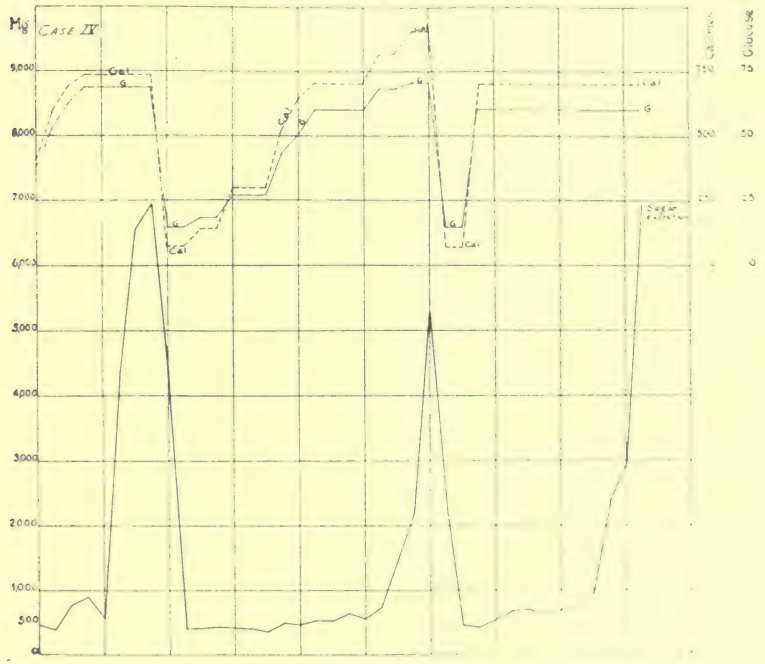


CHART 4.

abnormal quantities of sugar, was a sudden one. [Comment on the cause of the break may be deferred.]

Case 4.—(Mr. M.) A very severe case of diabetes mellitus. The results obtained with this case tend to confirm in a most striking manner those obtained with the above three cases, in that the sugar eliminated in the urine did not increase uniformly with the gradually increasing diet; but that there was a critical point at which a sudden and abnormally high excretion of sugar occurred in the urine.

Case 5.—(Mr. C.) Age 42 years. Weight 52 kilos. This patient's diet was built up gradually from 415 calories with G = 36 gm. to 1,295 calories

with G = 107 gm., while the total sugar eliminated in the urine every 24 hours remained practically constant (except on 1 day following a day of low excretion). In this case the diet at no time reached the point where a real critical acceleration of the sugar excretion occurred. The sugar excreted on the diet of 1,295 calories with G = 107 gm. was no higher than that excreted on the diet of 415 calories, for which G was 36 gm. This patient was neither in caloric nor nitrogenous equilibrium on the diet given, but left the hospital to continue treatment at home so that the chart was interrupted

Case 6.—(Mr. A.) A poorly nourished young man of 25 years. Weight 44.1 kilos. A severe case of diabetes mellitus. This patient came into the hospital with much sugar, acetone, and diacetic acid in the urine, and

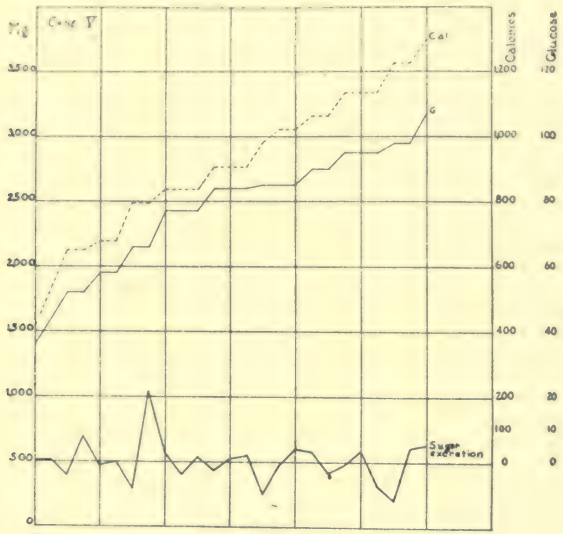


CHART 5.

a marked alkali deficit in the blood. He did not "desugarize" on a diet consisting of 400 gm. of "greens" and 1 liter of broth even after 4 days; and was fed more liberally till the acetone and diacetic acid disappeared. Then, following several dietary manipulations, his urine no longer gave a qualitative test for sugar. On a diet of 953 calories, for which G = 88 gm., the total sugar elimination was between 400 and 500 mg. per day; and on a diet of 1,837 calories, with G = 107 gm., the sugar excreted in the urine was only 330, 486, and 333 mg.

While the total quantity of sugar eliminated per day on a diet of 64 calories, with G at 14 gm. was practically the same as that eliminated on a diet of 1,993 calories (G = 114.7), it jumped suddenly to 1,485 and 9,800 mg. respectively when the diet was increased to contain 2,080 calories and the

equivalent of 121 gm. glucose. In this case observations were made of the relative effects of a high fat, high carbohydrate, low protein diet; and a high fat, high protein, low carbohydrate diet, each having the same value for *G*. These diets sufficed for maintenance. When the *G* of the diet was kept below a certain value, there was no significant change in the total urinary sugar excreted from day to day, regardless of changes in the relative quantities of carbohydrate and protein. In this case working laterally with maintenance diets, endogenous factors were reduced to a minimum.



CHART 6.

Case 7.—(Mrs. L.) Age 31 years. Weight 40 kilos. The experiment was begun with the patient on a diet of 830 calories, with $G = 49$. In this case the additions to the diet were made uniform. She was first kept on a constant diet of 25 gm. carbohydrate, 30 gm. protein, and 67 gm. fat, consisting of 400 cc. 5 per cent vegetables, 24 cellulose muffins, 3 eggs, 15 gm. butter, 100 cc. cream, 30 gm. bacon, and 10 gm. rice. For 11 days the sugar excretion was charted while the diet remained the same. During this period the excretion was not at first constant but it became so for the last 5 days (average 562 mg.). Thereafter additions were made always in the form of cream containing 16 per cent fat, with no other changes. The first addition consisted of 50 cc. cream. 3 days later, 25 cc. cream were

added and thereafter 25 cc. more each 4th day until six additions had been made, then 25 cc. cream every other day. In this case each fresh addition to the diet tended to cause a slight increase of the sugar excretion for that day followed by a return to the former level on the following day. These fluctuations were most marked early in the experiment and grew less as time passed. The average excretion was very constant at 600 to 700 mg. While no change took place in the total quantity of sugar eliminated per day (500 to 1,000 mg.), during the time that the glucose equivalent of the diet rose from 49 gm. to 70.3 *G* and the calories from 830 to 1,270, there was a sharp break in the sugar eliminated with the next addition of 25 cc. cream. With the diet aggregating 1,270 calories with *G* = 70.3 gm. the excretion ran 942 and 1,420 mg. on 2 successive days. The next addition was followed by excretions of 1,504 and 1,906 mg. Another addition was

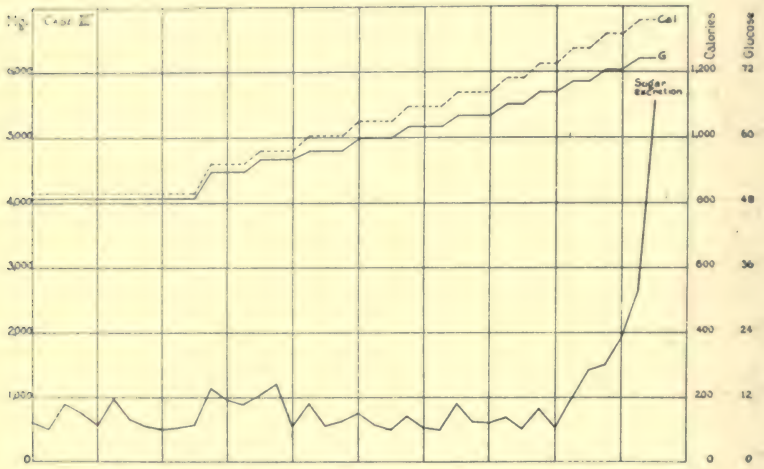


CHART 7.

then made and the excretion rose to 2,650, 5,568, and 6,300 mg. In this case it will be noted that when the total glucose equivalent of the diet was 68 gm. the excretion was still only 825 and 529 mg. on 2 days. The subsequent addition of food equivalent to 6.3 gm. glucose led to the excretion of approximately this quantity of glucose over and above the former average. There was, in short, a virtually complete excretion of all the glucose supplied in excess of a certain limit.

CONCLUSION.

Study of the curves obtained leads to the conclusion that individuals with severe diabetes, when brought into the non-diabetic status (or, as it is sometimes called, the "sugar-free" state) by

fasting or other more suitable adjustments of the diet, may then excrete small quantities of sugar not greater than those excreted by normal individuals under parallel conditions. The quantities in this series averaged between 10 and 15 mg. per kilo per day. As the diet is gradually increased stepwise at 1 to 4 day intervals, there is at first little or no *permanent* increase of the sugar excretion. The sugar excreted has remained entirely unaffected; or it has shown a definite but temporary acceleration with each new addition to the diet to be followed by a restoration of the former level; or it has shown a slight rising tendency from the start. But in any case the total permanent increase of the sugar excretion has remained slight or even unrecognizable until the total glucose equivalent of the diet has risen above a certain limit (which varies with the individual). Once this limit has been passed, further additions to the diet lead to rapid—even sudden—accelerations of the sugar excretions, out of proportion to any which have occurred before. The curve may then bend rapidly upward or show a true critical break.

This observation is in harmony with the well known conception of a clearly definable "tolerance limit" for glucose in diabetes; and that an "abnormal" sugar excretion may develop with critical suddenness when this limit is overstepped.

THE RÔLE OF THE URETERAL LYMPHATICS IN EXPERIMENTAL URINARY TRACT INFECTIONS

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In a previous article [1] the results of an experimental study to determine the routes by which infection could ascend from the bladder and involve the upper urinary tract were published. In that article these statements were made:

1. In experimental *B. coli* cystitis in dogs, blood stream infection rarely takes place.

2. Without stasis of urine, involvement of the upper urinary tract is rare.

3. With slight obstruction to complete emptying of the bladder, extension of infection to the upper urinary tract practically always occurs.

4. The involvement of the upper urinary tract almost uniformly takes place through the lumen of the ureter, and the ureteral lymphatics are rarely, if ever, the pathway of infection.

Since some of these statements are at variance with the reports of other workers on the same, or correlated, subjects, further evidence will be presented bearing on the rôle of the ureteral lymphatics as a pathway for infection involving the urinary tract.

URETERAL LYMPHATICS AS A PATHWAY FOR INFECTION WITHIN THE URINARY TRACT

Eisendrath and Schultz [2] described round-cell infiltration in the ureteral lymphatics after injecting *B. coli* into the bladder. Bauereisen [3] described the same findings in tuberculosis of the bladder. Stewart [4] explained the kidney infections following implantation of

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1. David, Vernon: Ascending Urinary Infections, Surg., Gynec. & Obst. **26**:159 (Feb.) 1918.

2. Eisendrath and Schultz: The Path of Involvement in Ascending Infections of the Urinary Tract, J. M. Research **35**:295, 1917.

3. Bauereisen, A.: Beitrag zur Frage der aszendierenden Nierentuberkulose, Ztschr. f. gynäk. Urol. **2**:132, 1910.

4. Stewart, L. F.: A Study of Ascending Infection of the Kidney Carried Out by the Transplantation of the Ureters Into the Intestines, Univ. of Penn. M. Bull. **23**:233, 1910-1911.

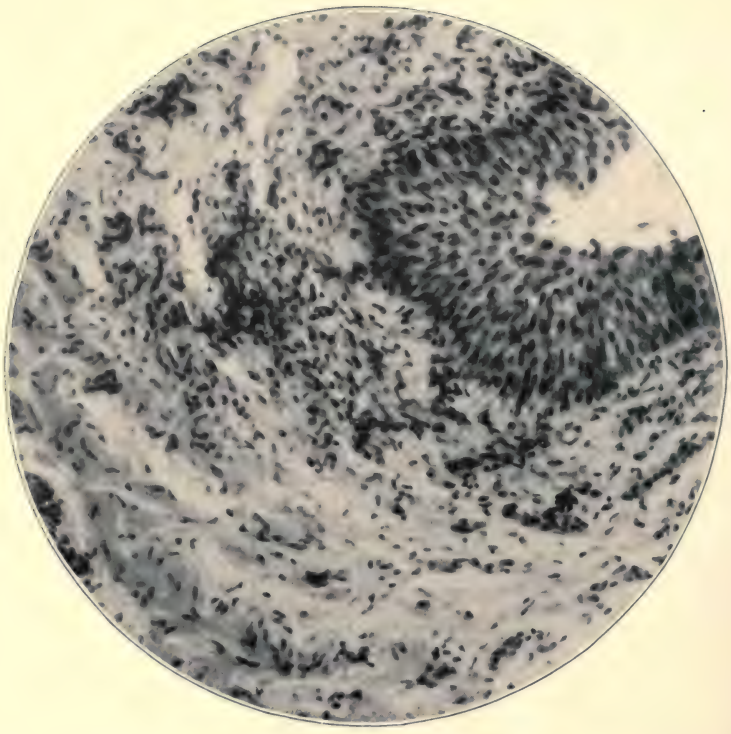


Fig. 1.—Round-cell infiltration in submucosa of ureter of control dog with sterile urine; × 230.

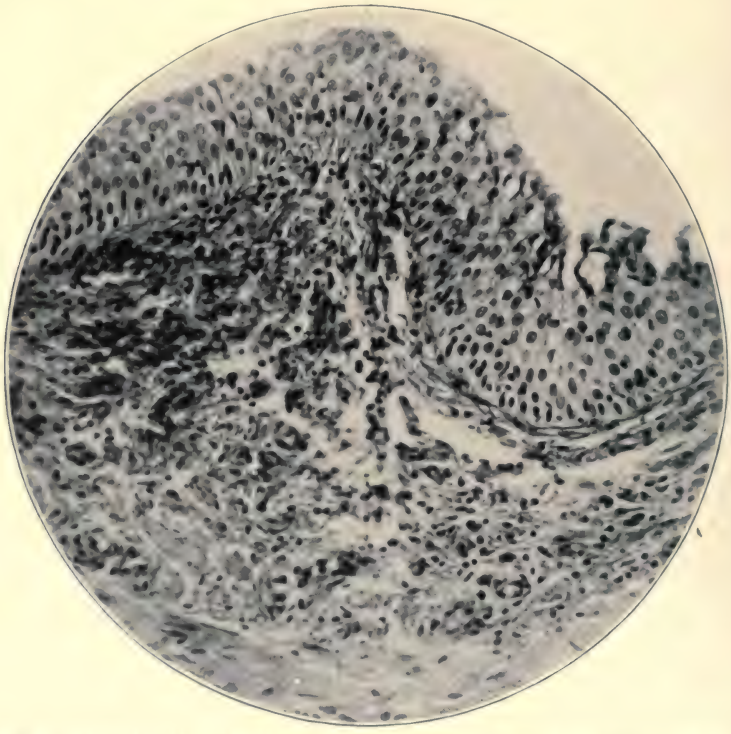


Fig. 2.—Round-cell infiltration in submucosa of ureter of control dog with sterile urine; × 230.

the ureters into the intestinal tract on the basis of an ascending infection through the lymphatics of the ureter. Franke [5] injected lymphatics running from the cecum and ascending colon to the kidney capsule and kidney pelvis. Helmholz and Beeler [6] injected cultures of a virulent organism, found in a rabbit kidney, into the bladders of a series of rabbits. They found pyelitis in fifteen of the animals, but there was evidence of cellular exudate in the ureteral lymphatics in only five. They concluded from this evidence that in some instances



Fig. 3.—Round-cell infiltration of the submucosa of the ureter; sterile cultures from the lumen of the ureter as well as from the macerated ureter; ×60.

the infection ascended by way of the ureteral lymphatics and in others through the lumen of the ureters. In another series of experiments on sixty-six rabbits, organisms of the *B. coli* group were injected intravenously, and pyelitis was found in only two animals. In some

5. Franke: Aetiologie der Coli-Infektionen, Arch. f. Anat. u. Physiol., 1910, p. 191. Ueber die Coliinfektionen der Harnwege, Berl. klin. Wchnschr. **48**: 1973, 1911.

6. Helmholz and Beeler: Infection of the Urinary Tract, Tr. Am. Pediat. Soc. **30**:195, 1918.

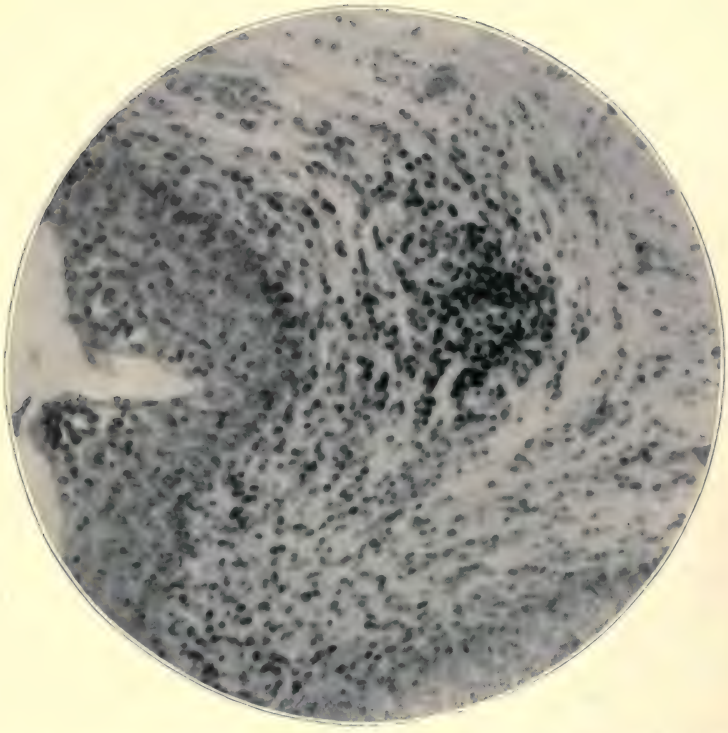


Fig. 4.—Round-cell character of infiltrate shown in Figure 3; ×230.

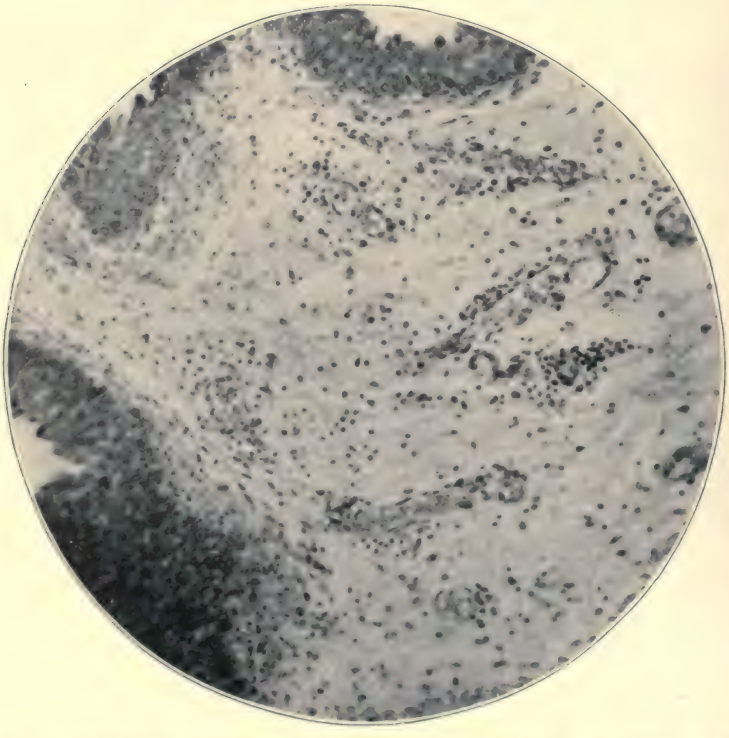


Fig. 5.—Bladder submucosa with polymorphonuclear infiltrate; ×130.

of the experiments a polymorphonuclear exudate was found in the subpelvic lymphatics.

Elaborate studies of the lymphatics of the bladder, ureters and kidney have been made by injection of the lymphatics with silver salts and Prussian blue (Mascagni, Teichman, Sappey, Krause, Gerota, Kumita, Stahr, Sakata). There is no doubt that a rather rich lymphatic system is found in the submucosa and muscularis of the bladder and ureter as well as in the periureteral fat, in the kidney

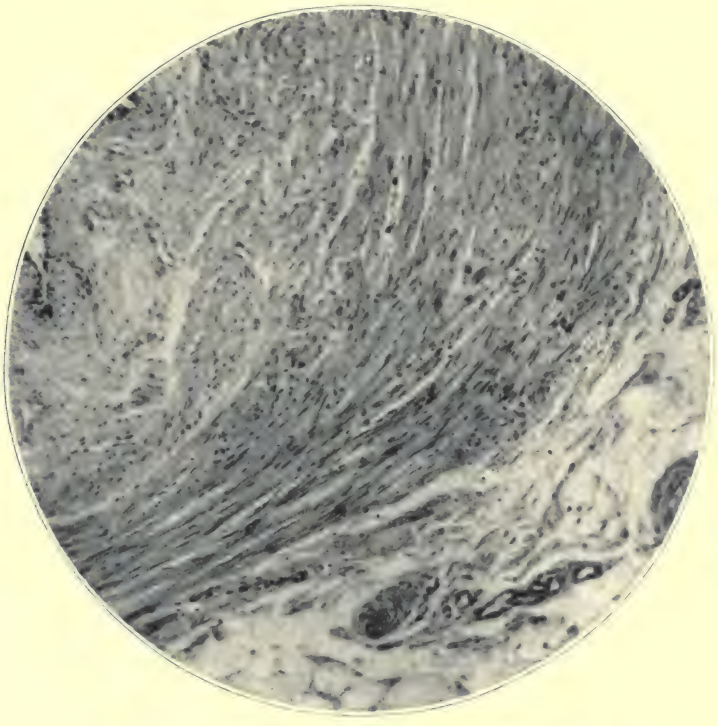


Fig. 6.—Ureter with no cellular infiltrate; × 130.

pelvis, in the parenchyma of the kidney and its fatty capsule. The lymphatics of the bladder, ureter and kidney have distinct communicating branches, but it is equally important to emphasize the fact that the drainage of the bladder, ureter and kidney is segmental into lumbar and hypogastric nodes and into the nodes along the renal vessels. The ready flow of lymph is not from the bladder through the ureter to the kidney, or vice versa.

Having established the premise that these organs have definite lymphatic systems and that involvement of them by inflammatory exudate has been described by several authors, we will herewith pre-

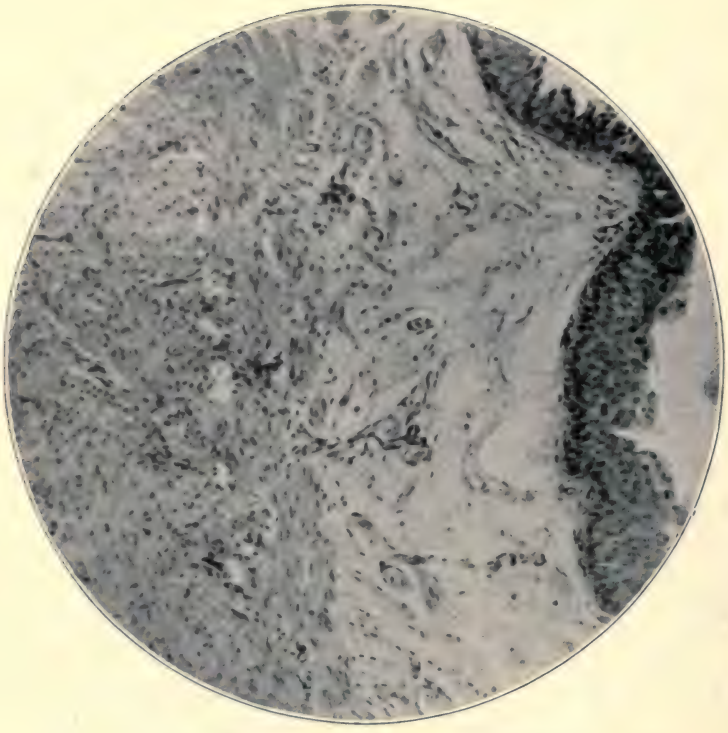


Fig. 7.—Kidney pelvis with no cellular infiltrate; × 130.

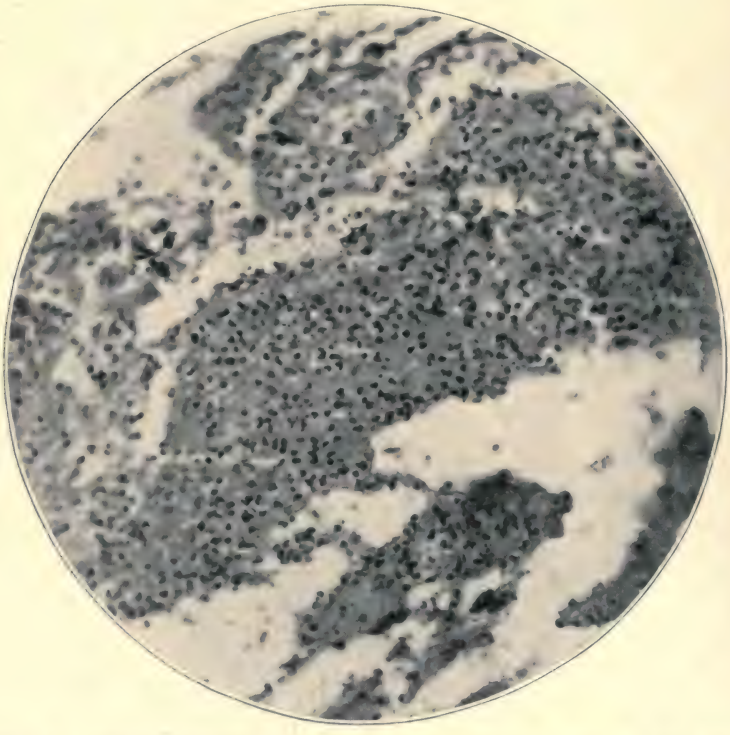


Fig. 8.—Polymorphonuclear exudate on peritoneal coat of the bladder; × 230.

sent our evidence to demonstrate that the occurrence of a cellular exudate in the lymphatics of the ureter and pelvis of the kidney does not necessarily indicate an infection of the urinary stream.

1. *Round-Cell Infiltration in Control Ureters with Sterile Urine.*—Control sections of the ureter in thirty-nine dogs have been studied microscopically, and at the same time the urine has been examined for bacteria by smears and cultures. Cellular infiltration in the lymphatics was absent in fourteen dogs, was present to a limited extent

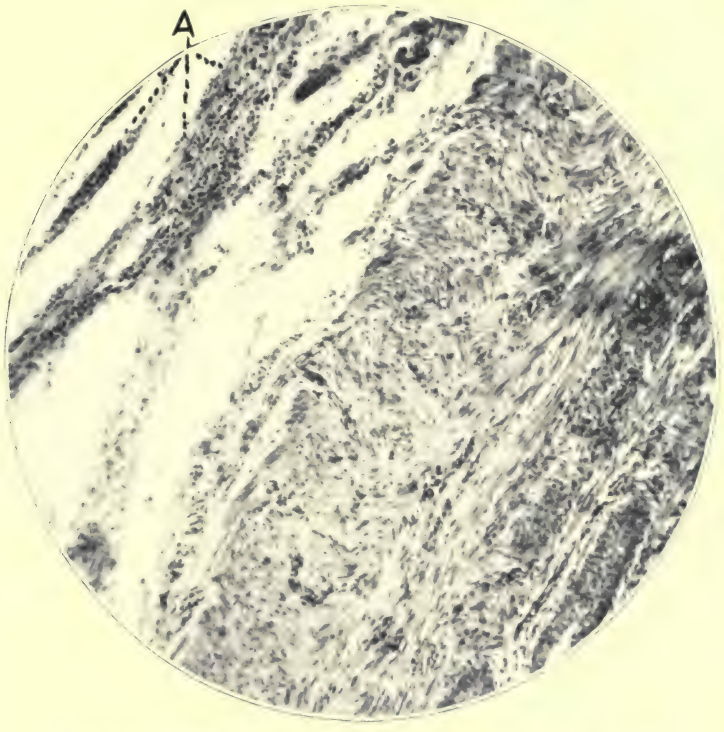


Fig. 9.—Ureter, showing perureteral exudate at *A*. ×90.

in ten, and in five dogs with sterile urine was present to a marked degree (Figs. 1 and 2). In rabbits the presence of round-cell infiltration in the ureter with sterile urine is even more common.

2. *Round-Cell Infiltration of the Ureter with Sterile Urine. Macerated Ureter Sterile.—B. coli* was injected into a dog's bladder, and the dog killed in thirty days. *B. coli* was still present in the bladder urine, but cultures from the ureters and kidney pelves were sterile. Microscopic examination of the tissue revealed a marked round-cell infiltration in the submucosa of the bladder and ureters (Figs. 3 and

4). A section of the ureter, which had been removed under aseptic conditions, was macerated in a sterile mortar, and cultures were made of the tissue extract. These were sterile. This experiment indicates that round-cell infiltration of the ureter is not synonymous with urinary stream infection, and that the presence of round-cell infiltration is not necessarily indicative of active infection.

3. *Ulcerative Cystitis with Ascending Ureteral Infection, but No Infection of the Ureteral Lymphatics or the Blood Stream.*—A num-

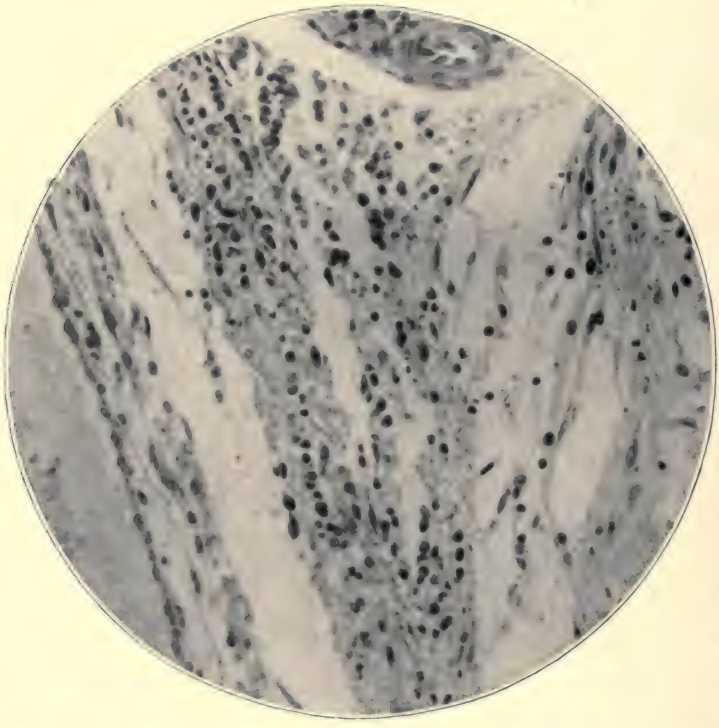


Fig. 10.—Polymorphonuclear type of exudate at *A* (Fig. 9); ×230.

ber of experiments on dogs were thus conducted: One ureter was ligated and divided to establish a hydronephrosis which acted as a control on blood stream infection, in which event it became converted into a pyonephrosis; the urethra was partially constricted by a band of fascia, but not sufficiently to prevent urination. *B. coli* was injected into the partially obstructed bladder; ulcerative cystitis developed with ascending infection involving the unobstructed ureter, from which *B. coli* was grown. Microscopically there was no cellular infiltration of any type in the ureteral wall though the bladder wall had a dense polymorphonuclear exudate throughout (Figs. 5, 6 and 7).

It is evident in these experiments that polymorphonuclear infiltration of the bladder does not extend to the lymphatics of the ureter and kidney pelvis with any rapidity, though ascending infection occurs early and readily through the lumen of the ureter.

4. *Cut End of Ligated Ureter Adherent to Infectious Material. Polymorphonuclear Infiltrate of the Periureteral Lymphatics. Hydronephrosis, Sterile.*—A few "happenchance" conditions arose in some experimental work which have a bearing on this problem. After liga-

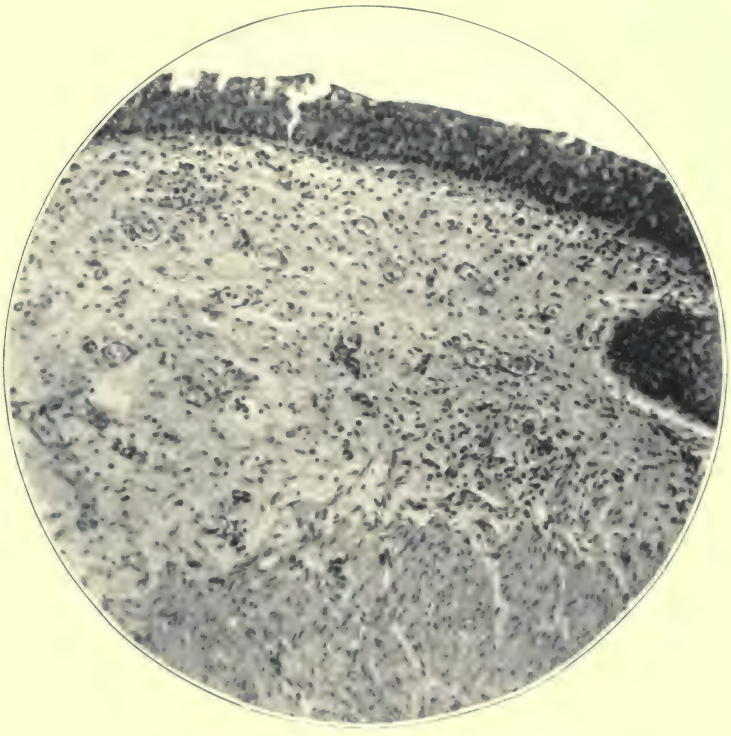


Fig. 11.—Polymorphonuclear exudate in submucosa of kidney pelvis; ×150.

tion and division of one ureter, some dogs came to necropsy with the peritoneal coat of the bladder covered with a plastic exudate due to accidental infection. To this exudate, the cut end of the ligated ureter was adherent. A microscopic study of the ureter demonstrated a rather marked polymorphonuclear exudate in the periureteral tissue reaching above the ureter to the subpelvic fat. However, in these particular experiments the hydronephrosis that developed above the divided ureter was sterile. A periureteral polymorphonuclear exudate with sterile urine was present (Figs. 8, 9, 10 and 11).

With these experiments in mind it seems clear that involvement of the periureteral lymphatics in round-cell or polymorphonuclear cell infiltration is not synonymous with urinary stream infection. Conversely, urinary tract infection occurs, ascending through the lumen of the ureters without involvement of the ureteral lymphatics.



Fig. 12.—Ureter above the gauze showing no infiltrate; × about 70.

URETERAL LYMPHATICS AS A ROUTE FOR EXTRA-URINARY INFECTIONS

The rôle of the lymphatics in conveying infection from the bladder to the ureters and kidneys has thus far been the object of our study. As a corollary, the importance of the ureteral lymphatics in conveying extra-urinary infections to the kidney will be considered.

It is theoretically possible in cases of pyosalpinx, pelvic peritonitis, acute appendicitis or appendiceal abscess that an inflammatory exudate coming in contact with a ureter might involve the periureteral lymphatics of the ureter and eventually spread along them and involve the pelvis of the kidney and kidney itself. This view has been

strongly urged by certain authors to explain the frequency of pyelitis in girl babies whose vaginal secretions have been found to contain *B. coli* soon after birth. In order to consider experimentally these presumptions, the effect of surrounding the ureter in a series of dogs and rabbits with a small piece of gauze impregnated with

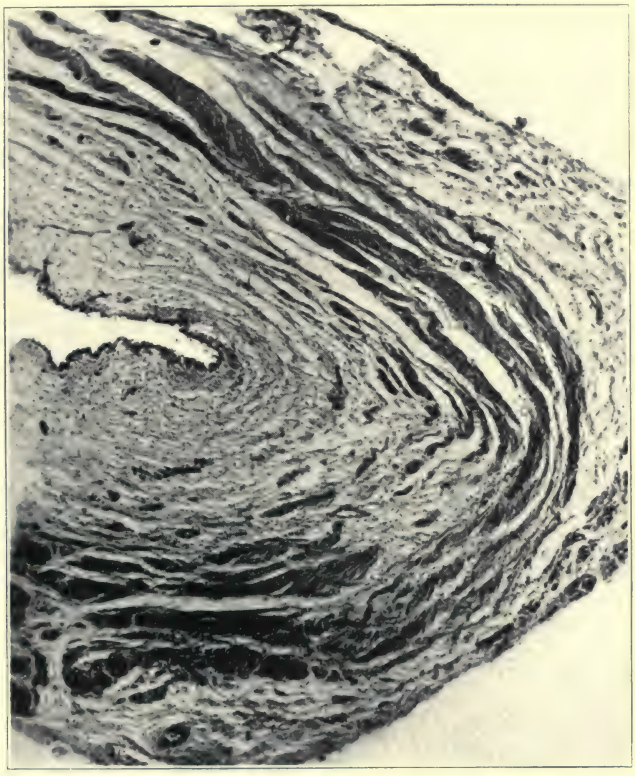


Fig. 13.—Kidney pelvis above the gauze showing no infiltrate; × about 70.

actively growing *B. coli* was studied. This simulates fairly well clinical conditions in which the ureter is in contact with infectious material.

In the first group of rabbits and dogs, a small piece of gauze was drawn under one ureter and saturated with *B. coli* after laparotomy. The opposite ureter was ligated and divided so that the ensuing hydronephrosis would act as a filter for blood stream infection. The control urine, taken before the experiment, was sterile in all of these animals. At postmortem, which occurred from seven to thirty days after the experiment, cultures of urine from both kidneys and bladder were sterile. *B. coli* was grown from the gauze around the ureter. Microscopically, in the region of the gauze, a dense polymorphonuclear

exudate was found around the ureter; but a few centimeters above the gauze, the ureter was free from cellular exudate. Sections of the kidney pelvis and kidney showed no cellular infiltration. On the opposite side, the hydronephrotic kidney and ureter were sterile and showed no cellular exudate (Figs. 12, 13, 14 and 15). The only exceptions to these microscopic findings were in several rabbits in which a few clumps of round cells were found in both kidneys, ureters and bladder. It seems improbable that these cells were related to



Fig. 14.—Ureter below the gauze showing no infiltrate; × about 70.

experimental conditions, as the cellular accumulations were isolated and corresponded to those found in the urinary organs of rabbits under control conditions.

It appears, therefore, that infectious material brought in contact with the periureteral lymphatics does not involve the urinary tract in infection by spreading along the ureteral lymphatics. There was no evidence of ascending infection involving the kidney pelvis or kidney parenchyma, nor was there any descending infection involving the bladder. In a few instances in dogs, the lumbar lymphatic nodes

in the region of the gauze were enlarged and in one instance they suppurated. This is striking evidence of the segmental drainage of the ureter.

In another group of experiments on rabbits and dogs, the ureter was ligated and divided, and the gauze around the ureter, saturated with *B. coli,* was placed near the proximal cut end of the ureter. At necropsy the cut end of the ureter was found embedded in the inflammatory exudate about the gauze. In some of these experiments,

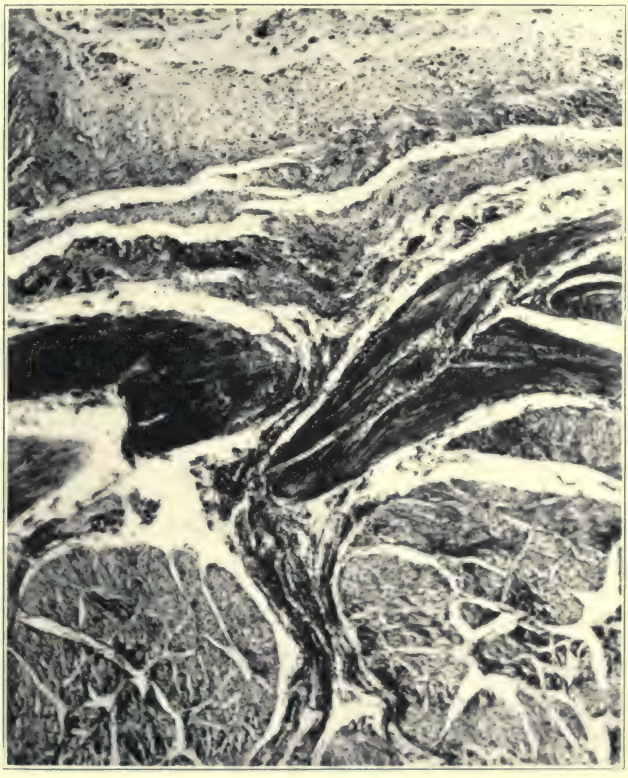


Fig. 15.—Bladder wall with the peritoneal coat showing no infiltrate; × about 70.

the bladder urine and urine from the opposite kidney as well as that from the developing hydronephrosis were infected with *B. coli,* which indicated that a blood stream infection had taken place. On the side of the pyonephrosis, in the submucosa of the kidney pelvis and the ureter as well as in the parenchyma of the kidney, groups of polymorphonuclear cells could be demonstrated microscopically, but there was no characteristic involvement of the spaces about the blood vessels where the larger lymphatics are located.

In other experiments of this type, the urine of the hydronephrosis was sterile as was the urine from the bladder and opposite kidney. There was no involvement of the periureteral lymphatics on the side where the gauze was implanted in the rabbit experiments; but in a few dogs, polymorphonuclear cells were found in the periureteral tissue as high as the pelvis of the kidney.

In experiments on two dogs and one rabbit, with the gauze in contact with the divided ureter, a pyonephrosis was found without periureteral lymphatic or blood stream involvement. The bladder and the kidney urine on the opposite side was sterile. In these instances, the infection probably extended along the lumen of the ureter by direct extension through its cut end.

In those experiments in which a cut end of a ligated ureter came in contact with infectious material, various results were recorded: (*a*) blood stream infection with infection of both kidneys and bladder; (*b*) involvement of the periureteral lymphatics above the point of contact with the infectious material but with no infection of the urinary stream, and (*c*) infection of the hydronephrosis above, with but little or no involvement of the periureteral lymphatics.

In no experiment, however, did it seem probable, after weighing all the evidence, that infection of the hydronephrosis took place by infection traveling along the ureteral lymphatics to the kidney.

CONCLUSIONS

1. Neither round nor polymorphonuclear cellular infiltration of the ureteral or subpelvic kidney lymphatics is synonymous with urinary tract infection, as we regard it clinically.

2. The lymphatics of the bladder and ureter play an unimportant rôle in the conduction of acute infections from the bladder to the kidney.

3. Infectious material brought into intimate contact with the periureteral lymphatics does not affect them to any considerable extent and infection of the urinary stream does not result.

4. An involvement of the periureteral lymphatics may occur when the cut end of a divided ureter, which has been ligated, comes in contact with infectious material; but it is improbable that infection of the kidney pelvis by this route occurs.

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FIVE HUNDRED AND THIRTY-FIVE NORTH DEARBORN STREET
CHICAGO

THE PRESENCE OF SUGAR IN THE URINE OF NEW BORN INFANTS BEFORE THE INTAKE OF FOODS*

FRANCES MILLIKIN

CHICAGO

Czerny and Keller [1] mention that Mesnil found sugar occasionally in the urine of infants during the first few days of life, and Nothman [2] found sugar in the urine of premature infants. The presence of sugar was shown by reduction tests, and in the case of Nothman the osazone was prepared which indicated the presence of lactose. By using the Benedict-Osterberg [3] method, Greenthal [4] showed that the urine of all infants contains sugar. This would be expected from the results obtained by Benedict, Osterberg and Neuwirth.[5] These authors found determinable quantities of sugar at all times, both in the urine of normal dogs, and those fasting for four-day periods, and in the urine of adults.

Thanks to the kindness of Dr. Sauer, we were able to obtain the urine of a series of new born infants for sugar determination.

The urine was collected during the period preceding the first feeding. None of the births was difficult, so that the results cannot be vitiated by the transitory traumatic glycosuria described by Hoeniger.[6] The method employed for the determination was that described by Benedict and Osterberg,[3] which has the important advantage of eliminating the possibility of the interference, in the determinations, of albumin, which occurs in the urine of the newly born. In all instances, duplicate determinations were made, all of which agreed very closely. In every case the urine was strongly acid to litmus. The first five cases are not recorded in the tables. They showed 0.036, 0.034, 0.072, 0.05 and 0.136 per cent. sugar, respectively.

Table 1 shows the quantitative determinations of sugar in the urine of individual infants. In Table 2, combined specimens from two or three infants, as indicated, were used. The amounts of urine per individual varied between 6 and 34 c.c. The amounts of total urinary

* Received for publication, Feb. 5, 1921.

* From the Otho S. A. Sprague Memorial Institute of the Children's Memorial Hospital.

1. Czerny and Keller: Des Kindes Ernährung, etc.
2. Monatschr. f. Kinderh. **8**:377, 1909.
3. Benedict and Osterberg: J. Biol. Chem. **34**: 195 (April) 1915.
4. Greenthal: Am. J. Dis. Child. **20**:556 (Dec.) 1920.
5. Benedict, Osterberg and Neuwirth: J. Biol. Chem. **34**: 217 (April) 1915.
6. Hoeniger: Deutsch. med. Wchnschr., **1**: 500 1911

sugar for the collection period ranged from 1.722 to 27.948 mg., with an average of 9.536 mg. The concentration of sugar in Tables 1 and 2 varied between 0.027 and 0.166 per cent., with an average of 0.078 per cent. Too much importance should not be attached to averages for so few cases.

Benedict, Osterberg and Neuwirth [5] found sugar in the urine of adults collected for two hour periods in the morning before food was taken, in concentrations of from 0.04 to 0.16 per cent. for one subject and from 0.03 to 0.049 per cent. for another. Greenthal [4] showed sugar to be present in the urine collected for eight hour periods from infants on ordinary diets in concentrations of from 0.045 to 0.092 per cent. The twenty-four hour urine of a normal adult on ordinary diet contained from 0.042 to 0.22 per cent sugar, that of another from 0.04 to 0.126 per cent.

TABLE 1.—Quantitative Determination of Sugar in Urine of Infants

No.	Name	Amount	Sp. Gr.	Total Sugar	
				Per Cent.	Mg.
1.	Dixon	7 c.c.	1.011	0.078	5.467
2.	Keck	6 c.c.	1.007	0.028	1.722
3.	White	17 c.c.	1.006	0.027	4.607
4.	Brewster	34 c.c.	1.003	0.082	27.948
5.	Koran	9 c.c.	1.010	0.061	5.540
6.	Brown	9 c.c.	1.012	0.043	3.906
7.	Trueblood	10 c.c.	1.012	0.051	5.100
8.	Nein	15 c.c.	.....	0.140	21.420
9.	Foster	15 c.c.	1.016	0.100	15.000
10.	Perry	17 c.c.	1.010	0.072	13.308
11.	Farnsworth	11 c.c.	1.010	0.128	14.102
	Average	13.6 c.c.	1.009	0.073	10.738

In Table 3 two determinations of the unfermentable sugar are given. There is sufficient reason to accept the fermentable sugar as glucose, but the nature of the unfermentable sugar is not known. In this table the unfermentable sugar was present in lower percentages (12.6 and 30.8 per cent.) than in the urine of infants and adults on regular diets or in the urine of adults during the fasting period of the day. For example, in urine collected from adults during the two-hour period in the morning before food was taken, Benedict, Osterberg and Neuwirth found unfermentable sugar to constitute from 57 to 73 per cent. (at one time falling as low as 36.3 per cent.) of the total urinary sugar for one subject and from 41.8 to 71.0 per cent. for the other. For adults on mixed diets they found the twenty-four hour urine

specimens to contain unfermentable sugar constituting from 40 to 65 per cent. of the total urinary sugar. Greenthal [4] demonstrated that of the total urinary sugar of infants on ordinary diet, from 38 to 49 per cent. was unfermentable sugar.

TABLE 2.—Quantitative Determination of Sugar in Combined Specimens of Urine

No. Name	Amount	Sp. Gr.	Total Sugar Per Cent.	Total Sugar Mg.
12. Horne ..... 13. Wells	23 c.c.	1.009	0.073	16.88
14. Pratt ..... 15. Bang ..... 16. Jones	25 c.c.	1.011	0.082	20.55
17. Friedlander .... 18. Benedict	21 c.c.	1.014	0.080	16.926
19. Blair ..... 20. Woodburn	30 c.c.	1.015	0.050	17.4
21. Morgan ..... 22. Morganson	12 c.c.	1.016	0.166	19.92
Average	11 c.c.	1.013	0.090	8.334
Average of Tables 1 and 2....	12.3 c.c.	1.011	0.078	9.536

The results of this series of urinary sugar determinations show that sugar does constantly occur in the urine of newly born infants before food is taken, and that it is present in about the same concentrations as in the urine of normal adults before food is taken in the morning.

TABLE 3.—Determinations of Unfermentable Sugar

No. Name	Total Sugar Per Cent.	Total Sugar Mg.	Fermented Sugar Per Cent.	Fermented Sugar Mg.	Unfermentable Sugar Per Cent.	Unfermentable Sugar Mg.	Total Sugar Per Cent.
4. Brewster 0.082		27.948	0.0718	24.416	0.0103	3.532	12.6
12. Horne ..... 13. Wells	0.073	16.88	0.051	11.69	0.021	5.191	30.8

The range of concentrations can easily explain why sugar is sometimes found in the urine of the newly born by the usual copper reduction tests. For instance, Miss Felcher, working with Dr. Woodyatt,[7]

7. With the permission of Dr. Woodyatt these data are published.

found that Haines' test gives a positive reaction readily with a concentration of sugar in the urine exceeding 0.07 per cent when 5 c.c. of the reagent and 2 c.c. urine are used.

As in the case of adults, as demonstrated by Benedict, Osterberg and Neuwirth, and of infants, as shown by Greenthal, this urinary sugar can be separated into a fermentable and a nonfermentable portion.

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FIVE HUNDRED AND THIRTY-FIVE NORTH DEARBORN STREET
CHICAGO

THE NON-SURGICAL TREATMENT OF PYLORIC STENOSIS *

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EVANSTON, ILL.

The treatment of pyloric stenosis is a topic which is still very much under discussion. This is mainly due to the fact that some clinicians advocate immediate operation while others get excellent results in many cases without such a radical procedure. Good results are often obtained without surgery because the spasm frequently relaxes when certain dietary measures are instituted. Operation, soon after the diagnosis is made, doubtless subjects many infants to unnecessary operation. On the other hand, persistence in unsuccessful feeding may increase the surgical risk.

The statement occasionally made by some pediatricians that the diagnosis of pyloric stenosis is synonymous with indication for operation is not in accord with the opinion held by the foremost surgeons in this field. Thus Downes,[1] points out very clearly that medical treatment is justifiable for a period of from seven to ten days, provided the patient does not lose too much weight (20 per cent. of the birth weight is the maximum weight loss he allows). Of the 163 cases of pyloric stenosis seen by Strauss [2] only 107 patients (65 per cent.) were operated on. He treated the remaining fifty-six medically. He says: "Not a single one of the medically treated cases returned for operation."

Most workers agree that the Fredet [3]-Rammstedt [4] operation, or a modification, is the best surgical procedure in those cases that fail to yield to proper medical treatment. It is true that some surgeons recommend immediate operative interference as soon as the symptoms of pyloric stenosis become manifest. Thus it has happened that on operation no tumor was found.[5] The abdomen was closed without further interference. Such a procedure would be perfectly legitimate if it had been shown that nonoperative treatment could only be successful in a small number of cases. But such is not the case. Besides the results

* Received for publication May 7, 1921.

* From the Pediatric Department of the Evanston Hospital and the Otho S. A. Sprague Memorial Institute Laboratory of the Children's Memorial Hospital, Chicago.

1. Downes: J. A. M. A. **75**:228 (July 24) 1920.
2. Strauss: J. A. M. A. **75**:232 (July 24) 1920.
3. Fredet: (Dufour and Fredet), Rev. de chir. **37**:208, 1908.
4. Rammstedt: Med. Klin. **8**:1702, 1912.
5. I do not wish to discuss here the appearance and disappearance of tumors which have been observed at the time of laparotomy.

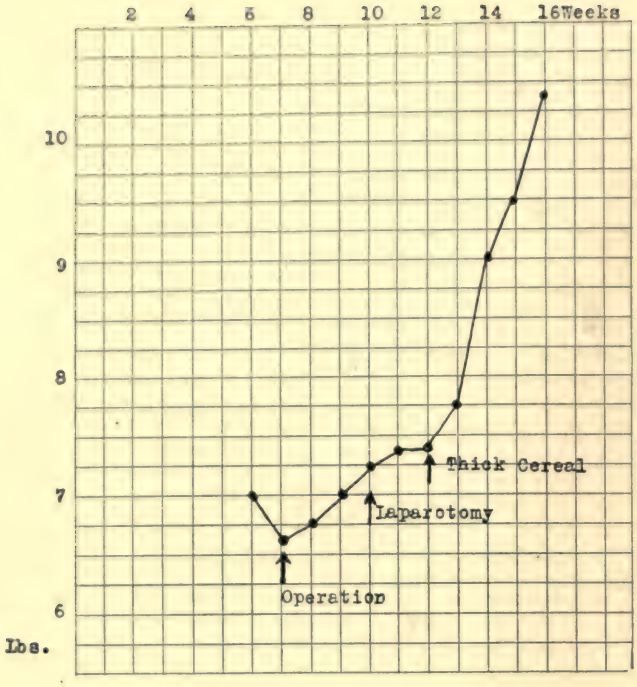


Fig. 1.—Case 1 (31). Postoperative adhesions.

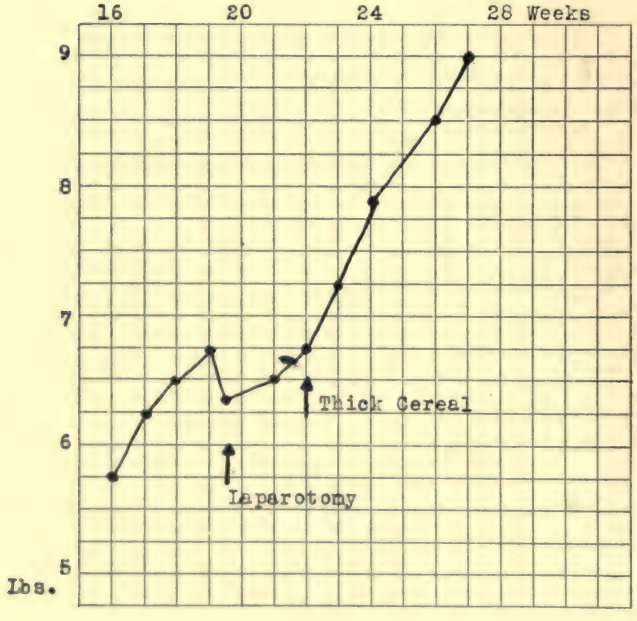


Fig. 2.—Violet S. Postoperative adhesions.

of Strauss, those of Heubner,[6] Ibrahim,[7] Porter,[8] Sedgwick,[9] and among others my own lend very strong support to the statement of Downes in regard to the justification of medical treatment. Indeed, it would, perhaps, be more correct to say that, as long as the patient is doing fairly well an operation is only justifiable when medical treatment has proved of no avail. An experienced surgeon can do this operation with minimal risk. On the other hand, when done by one of insufficient experience, the mortality may be very high, even when the surgical risk was very small. I speak here from experience.

That nearly moribund cases are a bad risk for the surgeon as well as for the internist is self-evident. Of the 175 patients operated on by Downes thirty died. Eighteen died from collapse shortly following operation—all were in extremely poor condition. Four others had intestinal complications, five developed peritonitis, and the remaining three died of hemorrhage. Twenty-two of his patients continued to vomit in moderate degree, and in sixteen others the vomiting occurred in large amounts. Various surgeons warn of the danger of incising the duodenum with a resulting peritonitis. Lewis [10] cautions that opening of the incision has occurred not infrequently when made in the linea alba. Of the twenty-two patients operated on by Hill,[11] two had to have the abdomen reopened. In one of the cases the opening was inadequate, in the other, the wound broke open on the sixth day and on re-operating adhesions were found.

Two cases with severe symptoms of postoperative obstruction in which the Fredet-Rammstedt operation had been performed by skilled surgeons are of interest; so is a case in which the operation was inadequate as all the symptoms of pyloric stenosis persisted for weeks until thick cereal feedings were substituted for the breast milk feedings.

REPORT OF CASES

Case 1.—(31) Alene T., was born Aug. 29, 1920, weighing 7 pounds, 12 ounces. When seen the first time at the age of 6 weeks the vomitus contained blood. On trying thick cereal feedings for a week, the vomited material contained more blood. The Strauss modification of the Rammstedt operation was performed. The result was gratifying, the vomiting ceased and the child gained in weight on breast milk feedings. On the sixth day, the little patient developed a cold with frequent coughing and sneezing. On the eighth day the nurse noticed that the stitches had sloughed and that the wound had partly opened with a partial evisceration. The intestines were immediately replaced and the wound closed. There was no rise in temperature, and after a few

6. Heubner: Lehrbuch der Kinderheilkunde, Ed. 3, Vol. 1, p. 218.
7. Ibrahim: Die Angeborene Pylorusstenose, Berlin, 1905. Ergebnisse d. Inn. Med. and Kinderh. **1**:208, 1908.
8. Porter: Arch. Pediat. **36**:385, 1919.
9. Sedgwick: Journal Lancet **37**:437, 1917.
10. Lewis: J. A. M. A. **75**:232 (July 24) 1920.
11. Hill: J. A. M. A. **75**:232 (July 24) 1920.

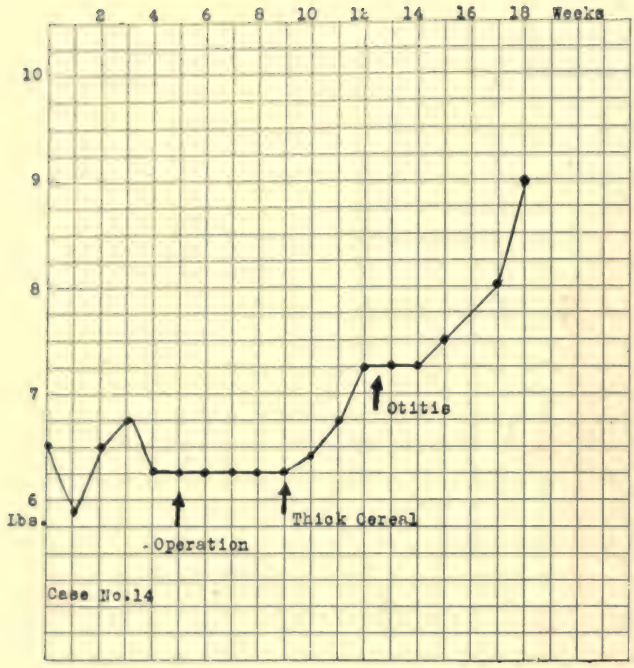


Fig. 3.—Case 7 (14). Postoperative vomiting.

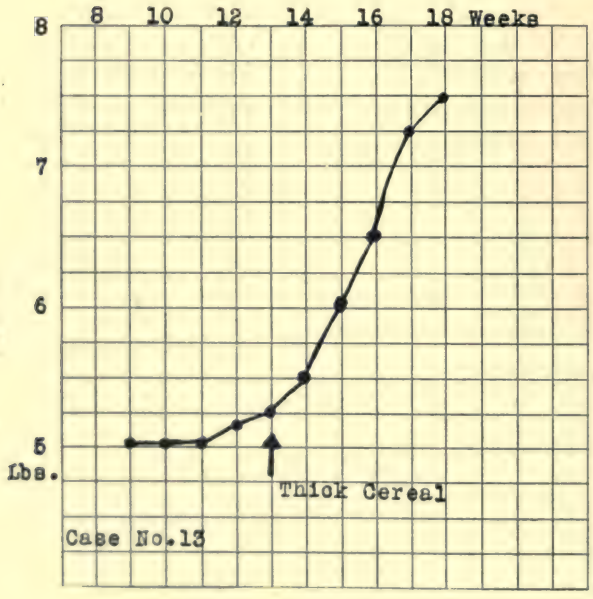


Fig. 4.—Case 13 (13). Rosie R. Tumor at necropsy.

weeks the child was sent to the Evanston Hospital. During the following weeks, there was more or less vomiting and the gain in weight was negligible. On the twenty-first day after operation the vomiting became projectile and incessant, the child became toxic and the bowels failed to move. The abdomen was soft, although intestinal peristalsis could be seen through the thin abdominal wall. The abdomen was reopened within twenty-four hours after the onset of severe symptoms. The skin incision was made parallel and lateral to the original one. A loop of small bowel had grown firmly adherent to the parietal peritoneum along the entire incision and was removed with difficulty. Laparotomy revealed numerous adhesions and bands. These were released and due to the moribund condition of the infant about 80 c.c. of uncitrated blood was given into the sinus. Recovery was very slow during the following two weeks. As more or less vomiting continued, barley flour paste made with breast milk was started two weeks after the laparotomy. The vomiting ceased at once and the child gained 7 ounces the first week and 1 pound the following week (Fig. 1). She is now 8 months old and weighs 17 pounds.

Case 2.—Violet S. was seen for the first time Jan. 19, 1921, at the age of 4 months, weighing 5 pounds and 12 ounces. The Rammstedt operation had been performed at the age of 6 weeks. There had been no gain in weight since the operation. The athreptic infant was getting condensed milk, had an enteritis and bronchitis with a temperature of 101 F. There was no vomiting. The child was given 50 c.c. of uncitrated blood intra-muscularly and fed breast milk. In the course of the following three weeks the infant gained 1 pound. February 7 severe vomiting, constipation and toxic symptoms rapidly developed. The abdomen was soft. Laparotomy was performed thirty-six hours after the onset of symptoms. The report of the surgeon, Dr. W. C. Danforth, follows:

> The child was extremely emaciated, the abdominal wall thin, moderately distended but without rigidity, showing very plainly distended coils of intestine. On opening the abdomen, many adhesions were present, omentum to abdominal wall at the site of the previous scar, light adhesions of coils of bowel one to another and in the ileum volvulus involving five inches of bowel twisted sharply on its mesentery. The strangulated area of bowel was very dark in color, but showed slight improvement in color on relief of the volvulus. In the mesentery thrombotic enlarged veins were seen. The intestine below the area involved in the volvulus was flattened, resembling coils of tape. As the strangulated bowel, after the application of hot laparotomy pads for a few moments, showed a slight improvement in color, and as the child's condition was desperate, it was determined to do nothing further and the abdomen was closed.
>
> The essentials of the convalescence are noted in Dr. Sauer's report. We were greatly and agreeably surprised at the recovery which the child was able to make.

The moribund infant was given 60 c.c. of uncitrated blood into the sinus. The recovery was slow during the following two weeks. February 27 the infant was given barley flour paste made as follows: Skimmed milk, 12 ounces; water, 4 ounces; barley flour, 2½ ounces; dextri-maltose, 1 ounce. Boiled one hour. Two and one-half ounces in four feedings; three ounces breast milk in three feedings. The infant gained 8 ounces in the following week, 10 ounces the week after that. She now weighs over 10 pounds (Fig. 2).

Case 3. (14).—Nancy B., was born Dec. 15, 1918, weighing 6 pounds 10 ounces. When seen the first time (Jan. 17, 1919) the infant had a temperature of 102 F. besides manifesting the typical symptoms of stenosis. A tumor could not be palpated, and the child was having from three to four stools a day (the family physician having ordered 15 drops of milk of magnesia before each nursing). Dextrose—Ringer's solution was given by rectum and subcutaneously. A catherized specimen of urine contained much pus, albumin and hyalin casts. The temperature varied from 101-105 F. The Rammstedt operation was performed January 24. An average sized tumor was found. She received breast milk until February 22 (four weeks) without any influence on the vomiting or weight. As the bladder condition had cleared up and the temperature was normal or subnormal, it was considered safe to begin thick cereal feedings. This was done cautiously during the following week. The

food was made as follows: Cow's milk or breast milk, 12 ounces; water, 8 ounces; barley flour, 2 ounces; dextri-maltose, 1 ounce. Boiled one hour. By the end of the first week of thick cereal feeding breast milk was discontinued entirely. She made an uneventful recovery (Fig. 3).

These three cases show what complications may occur after operation, even when operation is performed by skilled surgeons. These cases fall in line with observations published by other observers.

A few remarks about operative cases may not be amiss. Fluids should frequently be given subcutaneously or by rectum before or immediately after the operation. Blood, intramuscularly or into the sinus, is of unquestionable benefit in extreme cases. Breast milk should be given whenever available. If the supply is insufficient,

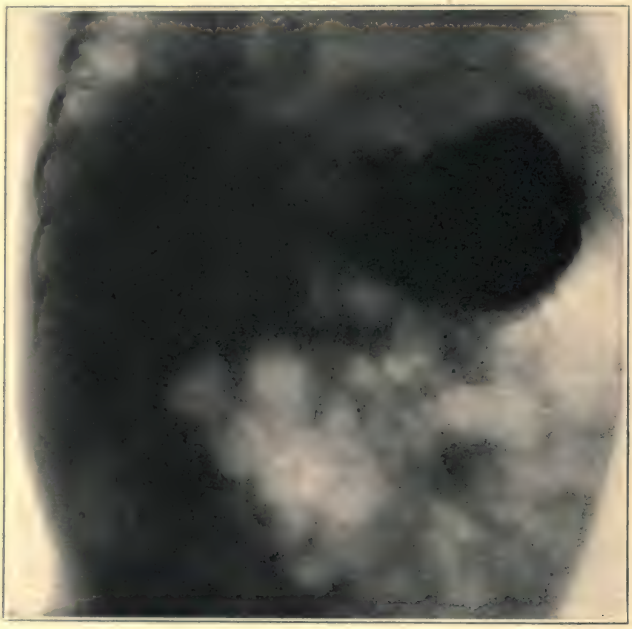


Fig. 5. Case 13. Series A.—Two hours after bismuth milk feeding (Strauss position).

albumin milk may be of value. Morgan [12] and Goldbloom and Spence [13] have detailed the postoperative management of Downes' cases.

Heubner, feeding breast milk to twenty-one infants with pyloric stenosis, had a mortality of 9.5 per cent., Sedgwick, using the same method in forty-four cases, lost but one patient. Of the eighty-three cases collected from the German literature by Ibrahim, the mortality

12. Morgan: Am. J. Dis. Child. **11**:245 (March) 1916.
13. Goldbloom & Spence: Am. J. Dis. Child. **19**:263 (March) 1920.

was 22.9 per cent. Finkelstein and Meyer [14] claim that no method of feeding will stop the vomiting in pyloric stenosis. On the other hand, Birk [15] advocated the use of cornmeal gruel. In his book a weight chart shows very conclusively that the infant gained rapidly as soon as this food was substituted for breast milk. Porter, using my method of cereal feeding, got rapid cures in ten successive cases. Moll [16] reports good results with cake-flour paste.

Thick cereal feeding is not a panacea for pyloric stenosis. There are undoubtedly cases which do not respond to the treatment as expected. Sometimes this is the fault of the physician and not the



Fig. 6. Case 13. Series A.—Four hours after bismuth milk feeding (Strauss position).

patient. Several of my patients (Cases 6, 8, 18) would doubtless have made more rapid progress if the proper precautions had been taken throughout the treatment. In favorable cases, the immediate decrease or cessation of vomiting and the rapidity of weight increase are very striking (see table).

---

14. Finkelstein and Meyer: Feer: Lehrbuch der Kinderheilkunde. Jena, Fischer. 1914.
15. Birk: (translated by Schlutz) N. Y. Rebman & Co., 1916.
16. Moll: Ztschr. f. Kinderh. **22**:147. 1919.

Success often depends on the proper preparation and administration of the thick cereal. It must be cooked thoroughly and be sufficiently thick to adhere to an inverted spoon. The influence of consistency is illustrated by the following two cases:

Case 4 (6).—Nelson L., weight 8 pounds and 4 ounces at birth, was seen for the first time at the age of 3 months. Various pediatricians had seen him and had tried various dietary measures. A tumor was palpable. One insisted that operation was necessary. Dr. H. F. Helmholz prescribed thick cereal feedings. There was no gain in weight and the projectile vomiting continued for ten days. On visiting the home it was found that the consistency of the food was



Fig. 7. Case 13. Series A—Four hours after bismuth milk feeding. (Very little has left the stomach.)

too thin. When this error was corrected, the vomiting immediately became less and the gain in weight was rapid. Peristaltic waves persisted for many weeks but all other symptoms of stenosis soon disappeared. The child now attends school and has never had a recurrence of symptoms. He is about 10 per cent. above the average weight for his height.

Case 5. (16).—Thomas N., weighing 7 pounds and 14 ounces when first seen at the age of 6 weeks, presented a typical picture of stenosis. As the milk mixture the physician had ordered was considered adequate, the only change made in the diet during the first month was the addition of six tablespoonfuls of farina. This made the food sufficiently thick and the vomiting became very much less. He gained 17 ounces during the first month of this feeding.

One part of cereal to seven parts of fluid (three of milk and four of water), boiled in a covered double boiler for an hour, usually makes a cereal of proper consistency. Such a food consists of about 15 per cent. cereal. Sugar and salt may be added. Most of the cases were started on a cereal as follows, and then the proportions were changed from time to time as occasion demanded: skimmed milk, 9 ounces; water, 12 ounces; farina or rice flour, 6 tablespoonfuls; dextri-maltose, 3 tablespoonfuls. Boil an hour or more in a covered double boiler until thick. The consistency of the food requires careful supervision.

A satisfactory way to give the food is to have the required amount of the prepared food in a cup immersed in a shallow saucepan con-

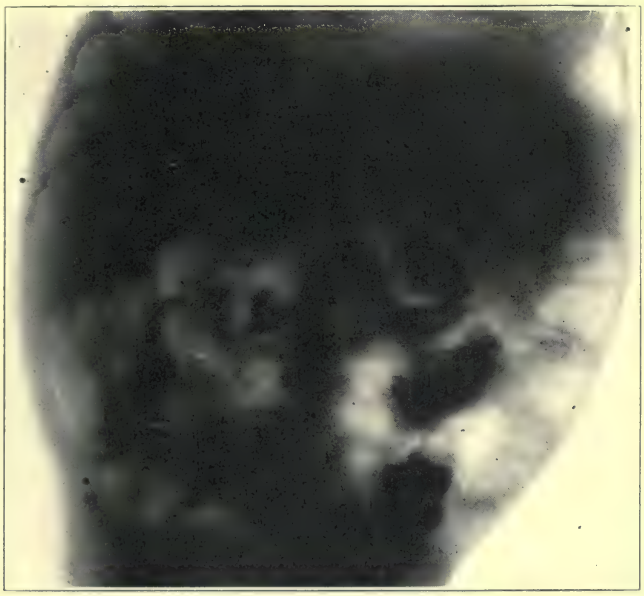


Fig. 8. Case 13. Series B.—Two hours after bismuth farina feeding (Strauss position).

taining hot water. By taking a little of the warm, thick cereal on the end of a narrow tongue depressor it can very easily be placed far back into the open mouth and scraped off with another tongue depressor. Porter recommends the use of a hygeia nipple with an enlarged hole. A great deal of patience is usually required the first few days, as the young infant often keeps the thick cereal in the mouth some time before swallowing it. At first, an hour or more may be required to give a feeding of a few tablespoonfuls, but this difficulty is soon overcome in most cases. Twenty-three of my patients were taken care of in the home.

From two to six tablespoonfuls of such cereal may be given six or seven times a day. When breast milk is available, one may try giving one or more tablespoonfuls of the cereal before the nursing or bottle breast milk feedings. If such a régimé proves unsuccessful, four thick cereal feedings and three breast milk feedings should be tried for a few days. If this fails to stop the vomiting and loss in weight, then all feedings should consist of the thick cereal. In severe cases, the latter procedure should be instituted at once. Any available breast milk should be added to the cereal replacing the cow's milk. The child should be placed on its right side for a few hours following the feeding. It must be handled very little and should rest on a medium soft pillow. In my last twenty-four cases the stomach was not washed out,

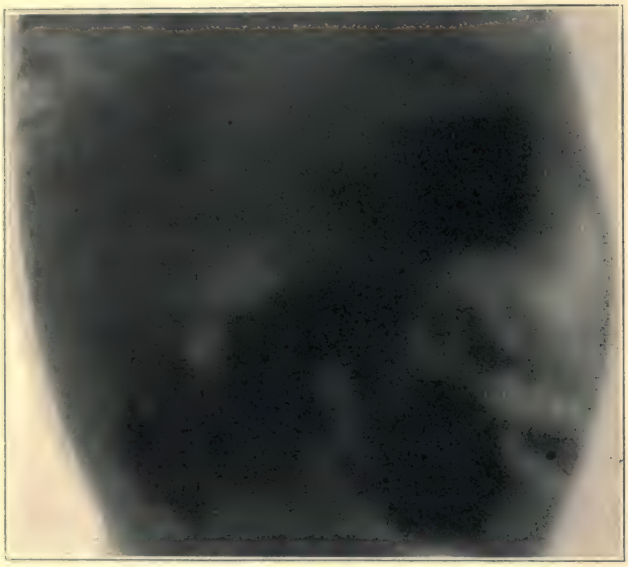


Fig. 9. Case 13. Series B.—Four hours after bismuth farina feeding (Strauss position).

in fact, very little fluid was given by mouth for the first few weeks. Water must be furnished at regular intervals, preferably by retention enema, by the Murphy drip method or by hypodermoclysis.

After thick cereal feedings have been persisted in for some time, liquid foods also pass through the pylorus. This interval varies in different cases—it usually requires at least from five to eight weeks before an appreciable amount of liquid food can be given without being vomited. The safest way to proceed when this time has apparently arrived is to substitute for one and later for several of the thick cereal feedings ordinary milk mixtures. If vomiting is not provoked in this

way the child can be put on a milk mixture without much further trouble in spite of persistent peristaltic waves and palpable tumor. During the past few years rice flour and barley flour have been used in place of or in conjunction with farina.

RESULTS IN THIRTY-FIVE CASES OF PYLORIC STENOSIS

No.	Name	Age in Wks.	Weight in Lbs. and Oz.	Weight 1 Mo. Later	Ounces Gain in 1 Mo.	Treatment	Result	Remarks
1	Ruth F.	6	5.0	6.12	28	C, Br.	Recovery	Hospital
2	Steph. W.	3	9.8	10.0	8	C, Br.	Recovery	Home and mother
3	Lloyd T.	13	7.8	9.14	28	C	Recovery	Home and mother
4	Harry H.	4	6.4	8.0	28	C	Recovery	Home and mother
5	Claren. T.	14	10.0	11.4	20	C	Recovery	Home and mother
6	Nelson L.	14	8.8	9.8	16	C	Recovery	Home and nurse
7	Robt. D.	10	6.8	7.12	20	C	Recovery	Home and mother
8	John A.	7	8.4	8.4	0	C, Br.	Recovery	Home and nurse
9	John B.	7	6.12	6.12	0	C	Improved	Hospital
10	Betty H.	4	5.4	6.4	16	C	Recovery	Home and nurse
11	Donald F.	3	6.9	6.12	3	C, 0	Recovery	Home and nurse
12	Betty M.	22	11.4	12.12	24	C	Recovery	Home and nurse
13	Rosie R.	12	5.0	7.2	34	C	Recovery	Hospital
14	Nancy B.	5	6.4	6.4	0	0, C	Recovery	Hospital
15	Betty C.	11	7.0	7.0	0	C, 0	Died	Hospital
16	Thos. N.	6	7.14	8.15	17	C	Recovery	Home and mother
17	Blain B.	12	9.8	11.2	26	C	Recovery	Home and mother
18	Norm. S.	12	7.15	9.7	24	C	Recovery	Home and mother
19	Virgin. D.	14	7.12	9.8	28	C	Recovery	Home and mother
20	Henry D.	5	6.8	9.14	54	C	Recovery	Hospital
21	Edw. P.	9	7.0	.....	..	C	Died	Hospital
22	James B.	13	8.12	10.8	28	C	Recovery	Home and mother
23	James C.	8	7.3	10.2	47	C	Recovery	Home and mother
24	Warren P.	7	6.8	9.10	50	C	Recovery	Home and mother
25	John W.	5	6.12	8.8	28	C	Recovery	Home and mother
26	Chas. S.	5	9.1	.....	..	C, 0	Recovery	Hospital
27	Ward P.	3	6.14	.....	..	C, 0	Recovery	Hospital
28	William M.	4	6.9	7.11	18	C	Recovery	Home and mother
29	Jerome R.	6	6.6	.....	..	C, 0	Died	Hospital
30	John Be.	9	10.8	12.4	28	C	Recovery	Home and mother
31	Alene T.	4	6.14	.....	..	C, 0	Recovery	Hospital
32	Everett W.	12	8.4	7.12	..	C	Recovery	Hospital
33	Wm. M.	5	6.9	7.12	19	C	Recovery	Home and mother
34	Clair W.	5	6.8	7.8	16	C	Recovery	Home and mother
35	Edw. N.	7	6.4	7.0	12	C	Recovery	Home and mother

C, thick cereal; 0, operation; Br., breast milk.

This report, covering thirty-five cases of pyloric stenosis, gives the results of all cases seen from 1914-1921 (see table). In a previous communication,[17] the first twelve cases were reported somewhat in detail. Twenty-two of the patients gained a pound or more during the first month. Twenty- four gained from the start. Eighteen were taken care of in the home by the mother. One of my thirty-five patients died while receiving thick cereal. This boy (Case 21) was admitted to the hospital when 9 weeks old weighing 7 pounds. The vomiting decreased as soon as the thick cereal was begun. There was no gain in weight. The child died suddenly on the ninth day. No necropsy was obtainable.

Operative Cases.—Five of the seven operated patients recovered.

17. Sauer: Arch. Pediat. **35**:385, 1918.

Case 6 (11).—Donald F. This case is of interest because peristaltic waves were never visible, nor could a tumor be palpated. The projectile vomiting began when the child was 2 weeks old. The vomiting occurred after most nursings. During the first few days, a tablespoonful or more of thick cereal was given before the nursings. As this was without effect, three thick cereal feedings were given, alternating with four nursings. The cereal was retained better than the breast milk, but the weight remained stationary. After another week, six cereal feedings were given, the breast milk being added to the thick cereal. In spite of the best nursing care, decrease in the vomiting and from one to three bowel movements a day, the child failed to gain. The vomiting occurred a few times a day but was very forceful. At operation, a tumor much larger and harder than usually encountered, was incised. The child made an uneventful recovery, being weaned at nine months.



Fig. 10. Case 13. Series B.—Four hours after bismuth farina feeding (stomach nearly empty).

Case 7 (14).—Nancy B. This was a case of pyelocystitis complicating the pyloric stenosis. When first seen, the baby was 5 weeks old and weighed 6 pounds 4 ounces. Her temperature was above 104 F. She was given fluids subcutaneously and by rectum and the Strauss modification of the Fredet-Rammstedt operation was performed a few days later (temperature 102 F.). The vomiting continued for four weeks after operation, although the cystitis had entirely cleared up (temperature normal for several weeks). Having received breast milk for four weeks after operation without a gain in weight, thick cereal feedings were gradually substituted. The vomiting ceased within three days and the child gained 18 ounces during the first month of thick cereal feeding. The operation in this case was inadequate (Fig. 3).

Case 8 (15).—Betty C., was 11 weeks old and weighed 7 pounds when admitted to the hospital. She was a patient of Dr. H. F. Helmholz. Her

admission temperature of 102 F. was probably due to "influenza." This was followed by a purulent otitis media and a subsequent bronchopneumonia. The vomiting was not very severe, but the roentgen ray showed that the food did not leave the stomach entirely at the end of four hours. As the weight remained stationary during the fifty days of thick cereal feeding, the Fredet-Rammstedt operation was performed under local anesthesia. The child vomited large quantities of blood after operation. She died three days later in spite of two blood transfusions. There was no necropsy.

Case 9 (26).—Chas. S., was referred by a pediatrician who had tried the thick cereal without result. The child was 5 weeks old and weighed 9 pounds 1 ounce (8 ounces less than at birth). He lost 9 ounces during the twelve days he was under my care. As he seemed to be suffering severe pain, the Strauss modification of the Fredet-Rammstedt operation was performed. Recovery was uneventful.

Case 10 (27).—Ward P., had projectile vomiting on the fourth day after birth. The vomiting subsided after a few days and the child regained his birth weight (8 pounds 2 ounces) on the sixteenth day when he was circumcised. That night the projectile vomiting was much increased and continued to be very forceful. Peristaltic waves were not very prominent. Roentgenograms taken four hours after a thick cereal feeding showed that very little food passed beyond the pylorus. The child lost 21 ounces in six days. In spite of lavage, bottle breast milk feedings and attempted duodenal feedings the weight continued to decrease rapidly. The Fredet-Rammstedt operation was performed on the twenty-second day. The tumor was not very large and not very hard. Recovery was rapid on breast milk.

Case 11 (29).—Jerome R., never regained his birth weight as the vomiting began a few days after birth. He was first seen when 6 weeks old, weighing 6 pounds 6 ounces. Thick cereal feedings had been attempted in the rural home by another pediatrician under rather adverse circumstances. When admitted into the hospital his general condition was poor. As he frequently refused to swallow the food and often appeared drowsy he was operated on eleven days after admission. He died rather suddenly two days after operation. No necropsy was obtainable. There were no manifest complications.

Case 12 (31).—Alene T., was admitted when 4 weeks old, weighing 7 pounds (5 ounces less than at birth). Thick cereal feedings were given for about a week, during which time she lost 6 ounces. As the vomitus frequently contained appreciable quantities of brown blood, she was operated on. She gained rapidly the following week, then a pharyngitis developed which was accompanied by other complications. (For subsequent details see above.)

Some clinicians and surgeons consider the roentgen ray an aid in diagnosis. Others use roentgenography to determine the degree of patency of the pylorus. Downes and Holt [18] consider it of no diagnostic value in typical cases. Strauss believes that a "fluoroscopic examination shows definitely not only if there is a congenital pyloric stenosis, but also if the patient should be treated medically or surgically. If from 70 to 80 per cent. of the bismuth milk goes through within four hours, there will undoubtedly be recovery, irrespective of any clinical symptoms or tumor." The estimation of the amount left in the stomach at the end of four hours requires a lot of experience, to say the least.

Our experience with the roentgen ray in this connection is of exceptional interest. The first case which was subjected to such examination

18. Holt: J. A. M. A. **68**:1517 (May 26) 1917.

is reported here in detail. This girl baby (Case 13) is undoubtedly the most interesting case of the whole series. Pictures were taken also of Cases 15, 16, 20, 27, 31 and 32. Pictures were taken with bismuth milk (Series A) and a few days later with bismuth thick cereal (Series B). The only change made in the formula was the addition of sufficient farina to make it thick. Roentgenograms were made with the patient in the position advocated by Strauss, also with the infant lying on the back. Roentgenograms were taken immediately after the feedings, also one, two and four hours later.

The first series shows that most of the bismuth milk was still in the stomach at the end of four hours, irrespective of the position of the child. In the second series, the same food, thickened with farina, quickly passed into the duodenum and the stomach was nearly empty at the end of four hours. This infant, according to Strauss' standard, would need operation. She was not operated on, but was given thick cereal and gained 34 ounces in thirty days (Fig. 4). She died of gonorrheal peritonitis some months later. A typical, cartilaginous hypertrophy of the pylorus was found, in spite of the fact that she had been absolutely free from feeding disturbance for a number of months.

Ibrahim,[19] Wernstedt,[20] Bloch,[21] Batten,[22] Simon,[23] and others have reported cases of known pyloric stenosis in which the patient died months or years after recovery without operation, but in which the cartilaginous tumor persisted.

Only two of the thirty-five cases included in this series failed to show the cardinal signs of pyloric stenosis. In Case 11 there were projectile vomiting, loss in weight, constipation, but at no time were peristaltic waves seen or a tumor palpated. At operation a typical cartilaginous tumor much larger than usual was found. In Case 14 peristaltic waves, projectile vomiting, rapid loss in weight, diarrhea and fever were noted. The former was due to the giving of milk of magnesia before the nursings, the latter to a pyelitis. At operation a typical tumor was found. In the remaining thirty-three cases typical peristaltic waves, projectile vomiting, loss in weight and constipation were present. In sixteen of the cases a tumor was palpated. Eighteen of the cases were on the breast when symptoms began. The cases were similar to those which had been subjected to operation before 1914.

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19. Ibrahim: Ergebnisse d. Inn. Med. f. Kinderh. **1**:208, 1908.
20. Wernstedt: Jahrb. f. Kinderh. **15**:674, 1907.
21. Bloch: Jahrb. f. Kinderh. **15**:337, 1907.
22. Batten: Lancet **2**:1511, 1899.
23. Simon: Jahrb. f. Kinderh. **23**:331, 1911.

When the diagnosis of pyloric stenosis is made the author tells the parents the nature of the ailment and that an operation may be necessary, but that thick cereal feedings will be tried for a short time to see whether the symptoms improve. The general condition of the patient, the stools, urine, vomitus, weight, food and feeding technic are closely watched. The presence of a palpable tumor or the size of the peristaltic waves have no bearing in making a decision. The weight, severity of vomiting, paucity of fecal material, complications and the general condition of the patient are the factors that determine how long thick cereal feedings may be tried. Fluids, preferably 6 per cent. dextrose Ringer's solution, are always given regularly by rectum or subcutaneously. If, after a thorough trial (from one to two weeks) the vomiting fails to subside, if the weight fails to increase, or if the general condition of the child gets worse, operation is resorted to. In some cases conditions may be such that early operation may be advisable, for example, when infants come under observation in a very emaciated state and fail to respond immediately to the treatment.

With so much still unexplained about the mechanism of the normal pylorus, it is impossible to give a satisfactory explanation of how the thick cereal passes into the duodenum in case of pyloric stenosis while the same food thinned out (to reduce its consistency) is promptly vomited. Moritz [24] found that in the adult solid food inhibited, and water stimulated gastric peristalsis. Schüle [25] found fluid foods to increase the motility of the stomach. If one watches the events with the fluoroscope after ingestion of bismuth thick cereal it is seen that the mass gravitates to the pylorus and that more material passes through in a given time than with bismuth milk.

SUMMARY

1. The use of thick cereal in thirty-five cases of pyloric stenosis brought about a rapid gain in weight in twenty-four cases and was effective in twenty-eight of the thirty-five cases.

2. The preparation and administration of the food are important.

3. Patients should be operated on if the thick cereal fails to bring about improvement in from one to two weeks.

4. Complications following operation are not infrequent.

5. Thick cereal feedings may be valuable when vomiting continues after operation.

24. Moritz: Ztschr. f. Biol. **42**:572, 1901.
25. Schule: Ztschr. f. klin. Med. **29**:73, 1896.

CONCLUSION

In cases of pyloric stenosis proper medical treatment, supported, when necessary, by proper operative treatment, will give the best results.

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OBSERVATIONS ON THE CAPILLARIES OF THE NORMAL INFANT*

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While seeking a method to determine the capillary blood pressure, Lombard [1] discovered that the circulation in the capillaries of the living person could be observed with a microscope. The skin was rendered transparent with either glycerin or cedar oil. Magnifications of from ten to twenty-five times were found best. For illumination he used either a Nernst lamp or daylight. Later Weiss [2] observed the capillaries of persons suffering from various diseases. In his method the electric light rays pass through a series of lenses before striking the area of skin to be illuminated; later, he used only one lense.[3] In determining capillary blood pressure, Danzer and Hooker [4] used merely a strong electric light and castor oil with their pressure apparatus and objective 3 and ocular 4 of the microscope.

The apparatus used to make my observations is very simple and not bulky. It consists of a small electric lamp (Fig. 1) with a strong convex lense made in the bulb.[5] The lamp is attached to the objective of the microscope by a metal attachment, in such a way that the light and the microscope can be focused on the same area. Objective 3 and ocular 2 were found best. The area to be observed is first cleaned with green soap, then with alcohol, and well dried before the glycerin or cedar oil is applied. The oil renders the skin transparent by penetrating the epidermis and so doing away with the light reflections from the uneven surface. In very young children, the oil can be applied directly without the preliminary cleaning. The third finger of the infant is strapped loosely, with a narrow strip of adhesive plaster to a wooden tongue depressor. With slight pressure on the knuckle the finger can be held quite firmly without interfering much with the circulation at the nail fold. Observations are made in the area about the middle of the nail fold. The infants were taken after the morning feeding, when they

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* From the Otho S. A. Sprague Memorial Institute Laboratory of the Children's Memorial Hospital.

* Read before the Chicago Pediatric Society, March 22, 1921.

1. Lombard, W. T.: Am. J. Physiol. **29**:335, 1912.
2. Weiss, E.: Deutsch. Arch. f. klin. Med. **119**:1, 1916.
3. Weiss, E.: Ztschr. f. Exper. Pathol. u. Therap. **19**:390, 1918.
4. Danzer, C. S., and Hooker, D. R.: Am. J. Physiol. **52**:136, 1920.
5. This lens was furnished by American Specialty Co.

would most likely sleep or be quiet. Needless to say, it is impossible to make observations when the child is irritable or very restless.

In 1892, Spalteholz [6] studied the distribution of the blood vessels in the skin by injecting the vessels with a colored gelatinous solution. Among his conclusions is the following statement:

> All the vessels which go to the papillae originate from the anastomizing vessels in the subcapillary plexus, and always in the form of small trunks, whose smallest branches run a short distance in the direction of the papillary rows, perpendicular under these, and run without further anastomoses and send their capillaries into the papillae. . . . (This coincides with my observations.) All arteries leading to the skin and their larger branches are the same in distribution and number in the new-born.



Fig. 1.—Microscope with lamp attached.

He makes no statement concerning the end capillaries of the skin in the new-born.

In 1919, Holland and Meyer [7] observed the capillaries in healthy normal children, and in those with exudative diathesis. They used the method described by Weiss. They describe and illustrate their findings in detail as follows:

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6. Spalteholz, W.: His Arch. f. Anat. u. Entwickelungsgesch. **1:** 1893.
7. Holland, M., and Meyer, L.: München. med. Wchnschr. **66:**1191, 1919.

In two-day old infants the end loops do not extend into the limbus (tissue between subcapillary plexus and skin surface), but remain in the region of the subcapillary plexus and extend up from there as short loops in the process of development. Arterial and venous limbs are clearly differentiated. Occasionally one finds a figure of eight loop. At one week regularity in distribution is seen. The short loops do not extend into the limbus. The venous limb is absolutely not differentiated from the arterial limb. (This differs from their findings at two days.) At four weeks the capillaries are of various widths, some are close together and with rich anastomosis, and some are more widely separated. Pronounced differences in length do not exist. The vessels project into the limbus.



Fig. 2.—The uppermost part of the pars papillaris cutis without the epidermis (Spalteholz).



Fig. 3.—Normal infant, 2 days old.

They describe the picture in the normal young infant as follows:

The width of the capillaries is from 0.03 to 0.05 mm. and length 0.15 mm. Difference in length and variation in width is seldom found, and then the difference is not marked. Well marked serpentine forms are seldom found, but, as a whole, there is a tendency to greater irregularity and serpentine forms. The vessels run straight, but not exactly parallel. Markedly twisted loops are seldom found. The arterial and venous limbs are almost always

well differentiated. In almost all children, the subcapillary plexus is clearly seen. Anastomosis one seldom finds among the end capillaries, but the subcapillary plexus is rich in anastomosis.

Later, Mertz[8] published his observations. He divides his results into three groups; first, comprising the children from 1 to 3 months of age; second, from 4 to 6 months, and third, those over 6 months.

In the first four or six weeks the loops are very short and project up from the ground substance into the limbus, more as broad bow shaped protrusions than as short loops. It is only the distal part of these loops that one can term the capillary loops. After several weeks there is found a better development of the capillaries and an extension into the skin along the edge of the nail fold. The age when this definite development occurs seems dependent on the development of the child. In the first three months, the capillary loops are from 20 to 60 microns long, are relatively not close together and anastomosis is seldom seen. In the following period, from 3 to 6 months, there is a rapid lengthening of the loops; they reach 200 microns. The loops are closer together and have greater anastomosis than before.



Fig. 4.—Normal infant, 7 days old.

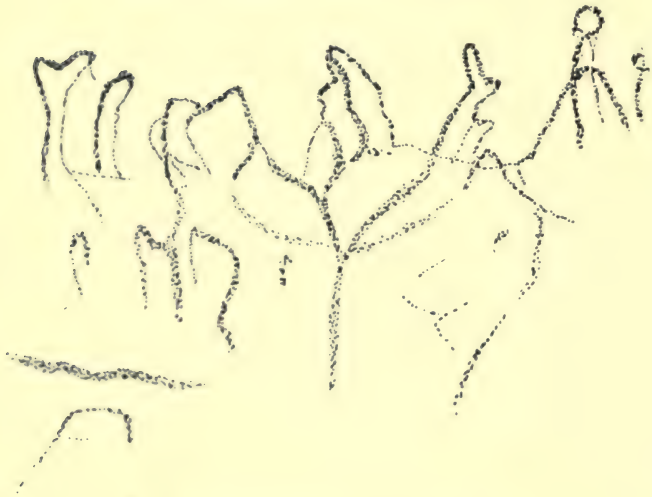


Fig. 5.—Normal infant, 3 weeks old.

8. Mertz, A.: Monatschr. f. Kinderh. **18**:13, 1920.

He describes a variation in length of the capillaries of children of the same age and in the same child on different fingers, this being related to the length of the nail fold; if it is pushed back, the loops are shorter than if it is grown further out over the nail.

My observations were made on infants at the Children's Memorial Hospital and at St. Vincent's Orphan Asylum. Only those infants were examined who appeared to be in good condition. No child with



Fig. 6.—Normal infant, 5 weeks old.

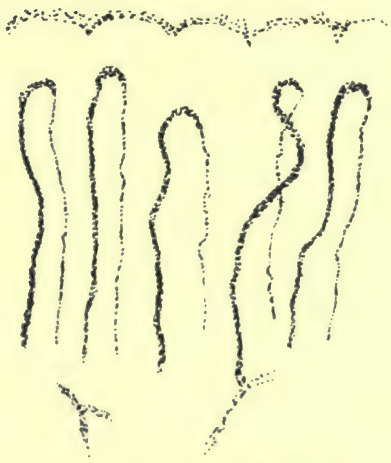


Fig. 7.—Normal infant, 9 months old.

an infection, however slight, with eczema, or in poor physical development was taken as a normal child. Many infants could not be observed as they became too irritable when an attempt was made to hold the hand absolutely quiet under the objective of the microscope. It was found most satisfactory to hold the infant in a sitting position in such a way that the hand could rest comfortably on the stage of the micro-

scope. Often the infant slept quietly during an entire observation. Drawings were made of those infants who held absolutely quiet for a relatively long time.

In infants 1 or 2 days old (Fig. 3) the capillaries appear as a fine network plexus lying close to the skin surface. This plexus can be seen extending back from the edge of the nail fold for quite a distance. Only the most distal vessels of this plexus can be called end capillaries. These are bow shaped, vary in length and height, and the arterial and the venous limbs can be differentiated. The arterial limb is hairlike



Fig. 8.—Normal adult.



Fig. 9.—Child with malnutrition, 10 weeks old.

and broadens gradually into the venous limb. There is no regularity in the distribution of these loops along the plexus margin. Occasionally, there is a short narrow loop. There is no anastomosis except in the subcapillary plexus.

At one week (Fig. 4) the capillaries begin to elongate and project out into the tissue between the plexus and the skin surface. In some the loops appear triangular, the angle appearing at the top and the base remaining broad. Other loops may be somewhat square. The

capillaries begin to appear more loop than bow shaped and they are slightly more regular in their distribution. There is no anastomosis between these and loops. When such seemed to be present it was found upon focusing that one loop was superimposed on another. The arterial and venous limbs are clearly differentiated.

At three weeks (Fig. 5) the loops are definitely formed and are separate from each other at relatively equal distances, one loop extending into each papilla. The loops vary in length and breadth, many loops being still quite broad and short. No anastomosis occurs except in the subcapillary plexus, that is, the vessels at the base of the loops. The arterial limb of the capillary vessel is hairlike and broadens out into the venous limb before turning back on itself. The venous limb often is slightly tortuous. The apparent anastomosis in Figure 6 was found to be superimposed vessels in which the two limbs were not in the same plane. There is little if any variation in the caliber of the different vessels.

At two months the capillaries are longer and the distance between the subcapillary plexus and the skin surface is also greater. The vessels disappear in the plexus, which appears as a pinkish haze with here and there a deeper red line, representing either a larger vessel or one closer to the surface.

At about three months, the capillaries are well formed; they are longer than those at two months. There are some spiral and figure of 8 shaped vessels. As the capillaries increase in length, they appear much closer together. From three months on the vessels increase in length until the long narrow loops of the adult type (Fig. 8) are reached. The subcapillary plexus shows less anastomosis at the base of the loops.

In general, it might be said that from birth the end capillaries begin development from small loops at the periphery of the subcapillary plexus. Until about the third month the development is very rapid. From then on there is not much change.

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